STUDIES ON ALVEOLAR MACROPHAGES IN THE PRESENCE OF PSEUDOMONAS ALGINATE AND ANTIBIOTICS.

by

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TO SARAH AND DUNCAN

DECLARATION

I confirm that the work in this thesis was conceived, planned and executed by myself.

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SUMMARY

Patients with cystic fibrosis often have chronic pulmonary infection caused by mucoid, alginate producing Pseudomonas aeruginosa. These bacteria are difficult to eradicate and are indicative of cystic fibrosis. The hypothesis that the production of alginate confers a selective advantage on the mucoid form of P.aeruginosa by impairing the host's immune response was examined. Alginate obtained from a mucoid strain of P.aeruginosa inhibited the phagocytosis of an isogenic non-mucoid revertant by rat alveolar macrophages. The phagocytosis of Staphylococcus albus and the binding of unopsonised bacteria were also inhibited in a dose-dependent fashion. Inhibition occurred when the macrophages were pre-treated with alginate and when the alginate was added with the bacteria to the macrophage monolayers. The inhibition was reduced when the macrophages were washed vigorously after alginate treatment and phagocytosis returned to control levels when the macrophages were re-incubated in normal serum after alginate treatment. The binding of antibody sensitised sheep erythrocytes (EA_G) to Fc receptors and the nonspecific uptake of latex particles was also inhibited by pseudomonas alginate in a dose-dependent fashion. Alginic acid, a polysaccharide purified from seaweed, which is structurally similar to alginate also inhibited the phagocytosis of non-mucoid P.aeruginosa by rat alveolar macrophages. This indicated that the observed effects of alginate were not due to bacterial toxins contaminating the material. Alginate inhibited the opsonisation of the non-mucoid revertant with a non-agglutinating immune serum. At

high alginate concentrations, the observed inhibition reached 100%. These results suggest that the alginate acts as a barrier, surrounding the macrophage or bacteria, to prevent the attachment of bacteria and other particles to the macrophage membrane and inhibit opsonisation. The importance of this impairment of the host's immune response by pseudomonas alginate is discussed.

Mucoid P.aeruginosa are rarely eradicated from the lung, even with extensive antibiotic therapy. One of the reasons for this may be that only low levels are attained in the cystic fibrosis lung. The further possibility that antibiotics may affect alveolar macrophages, in which function is already impaired by alginate was examined. Three antibiotics, azlocillin, ticarcillin and tobramycin, that are used in the treatment of P.aeruginosa lung infections in cystic fibrosis patients were studied at low concentrations for their effect on rat alveolar macrophages. Azlocillin, a semi-synthetic penicillin inhibited the phagocytosis of opsonised non-mucoid P.aeruginosa and the binding of antibody sensitised sheep erythrocytes when added with the indicator cells to the macrophage monolayers. However, no effect was observed when the macrophages were pre-treated with azlocillin for 30 minutes. Ticarcillin, a semi-synthetic penicillin that is less active as an antimicrobial agent in vitro than azlocillin, had no effect on alveolar macrophages at the concentrations used in this study. Tobramycin, a highly active aminoglycoside inhibited the phagocytosis of opsonised non-mucoid P.aeruginosa and the binding of unopsonised bacteria and enhanced Fc receptor expression when the macrophages were pretreated with antibiotic. These effects were dose, temperature and time-dependent. Inhibition of both phagocytosis and binding of EA_G was

observed when tobramycin was co-incubated with the macrophage monolayers and indicator cells.

Tobramycin and azlocillin had no effect on opsonised P.aeruginosa or sensitised sheep erythrocytes, except that the phagocytosis of EA_G was increased after treatment of the erythrocytes with tobramycin.

Culture supernatants prepared from macrophages treated with tobramycin inhibited the binding of EA_G to macrophage monolayers. The inhibition was directly proportional to the amount of supernatant added to the red cells and was greatest when the supernatant had been prepared from macrophages incubated with the antibiotic for one hour. When macrophage monolayers were incubated for one hour with supernatants from tobramycin treated macrophages subsequent binding of EA_G was increased. The supernatants also caused the agglutination of antibody sensitised erythrocytes. This activity was lost after the supernatants had been absorbed against formalised EA_G. The supernatants were further analysed by polyacrylamide gel electrophoresis. Those from antibiotic treated macrophages contained more material compared to control supernatants at two bands of approximately 45,000 and 66,000 daltons. It is proposed that the supernatants contain released Fc receptors and that tobramycin causes an increase in the shedding of Fc receptors from the macrophage membrane. The mechanism of action of tobramycin and the in vivo relevance of these results is discussed.

Studies on human peripheral blood monocytes were also carried out. Azlocillin enhanced the binding of EA_G to monocytes when co-incubated with the erythrocytes and monocyte monolayers while ticarcillin had no effect. Tobramycin inhibited the binding of EA_G to Fc receptors when added with the erythrocytes to the monolayers. When

results from different individuals were pooled, pre-treatment with tobramycin had no effect on Fc receptor expression. However when monocytes were taken from the same individual on different days, tobramycin inhibited or enhanced Fc receptor expression according to the individual studied. Therefore a longitudinal study may give different results than when results from different individuals are pooled. These results indicate the necessity for further work on human cells and their significance is discussed.

CONTENTS

CHAPTER 1

INTRODUCTION

1.0 CYSTIC FIBROSIS

Cystic fibrosis (CF) is currently the most common fatal hereditary disease of Caucasian populations. The incidence is one per 2,000 live births and it is inherited as an autosomal recessive trait. Clinically CF is characterised by elevated sweat electrolytes, chronic obstructive pulmonary infection and pancreatic insufficiency. However the main cause of death, is progressive pulmonary deterioration with characteristic intermittent exacerbations of chronic bacterial infection.

The earliest known reference to CF comes from the German folklore, which literally translated states "The child will soon die, whose brow tastes salty when kissed" (*Wood et al., 1976). In 1938, Dorothy Andersen distinguished CF from coeliac disease and described the destructive lesion of the pancreas associated with CF. Initially CF was considered to involve the pancreas as the sole target organ, but it soon became apparent that CF patients had a generalised disorder of mucus secretion. This was shown to affect most exocrine glands and other organs particularly the lung. Today, pancreatic insufficiency and its complications can be effectively overcome, by addition of pancreatic supplements to the diet. The high mortality of these patients is now a result of chronic pulmonary infection, and effective treatment is the main problem of CF today.

When CF was first recognised, few patients reached adolescence as the disease was invariably and rapidly fatal. With improved diagnosis and early patient care, 50% of all patients survive to 20 years of age (Warwick, 1982). This has resulted in an increasing adolescent and adult population, so that CF is no longer a strictly paediatric disease.

(* taken from)

1.1 The Bacteriology of Cystic Fibrosis

Staphylococcus aureus, Haemophilus influenzae and Pseudomonas aeruginosa are the three main pathogens that have been involved in CF pulmonary infection. However as survival has improved, a changing pattern of infection has been observed.

In 1946, Staph. aureus was the most common etiological agent isolated from the sputum of CF patients (di Sant 'Agnese and Andersen, 1946). With the advent of effective antistaphylococcal antibiotics, infection with Staph. aureus decreased significantly, while infection with P.aeruginosa began to increase (Mearns et al., 1972). The importance of Staph. aureus as a cause of mortality in CF patients has continued to decrease and is now minimal (Høiby, 1982). In 1963, Iacocca et al., reported that Staph.aureus remained predominant, but that the incidence of P.aeruginosa and H.influenzae were increasing. H.influenzae is also a significant pathogen, but can be treated effectively with appropriate antibiotics (McCrae and Raeburn, 1974).

P.aeruginosa is presently the predominant bacterial pathogen in CF, particularly in older patients and occurs in 70-90% of CF cases (Isles et al., 1984). Following primary colonisation with classic non-mucoid forms of P.aeruginosa, mucoid, alginate producing forms of the same micro-organism emerged and came to predominate with a corresponding increase in pulmonary deterioration (Doggett et al., 1966; Doggett, 1969; Hoiby, 1975). In 1982, it was reported that non-mucoid forms of P.aeruginosa appeared to be of no major significance, further indicating that the mucoid form is associated with a poor prognosis (Henry et al., 1982).

Although P.aeruginosa, Staph.aureus and H.influenzae are often isolated simultaneously from the same individual (Mearns, 1980), there is a lower prevalence of the two latter bacterial species in patients

with P.aeruginosa infection (Høiby, 1982).

A recent report has shown that the prevalence of P.cepacia in CF has increased from 10% in 1971 to 18% in 1981, while that of P.aeruginosa has remained unchanged. P.cepacia is uniformly resistant to all commonly used anti-pseudomonal agents and infected patients have greater impairment of pulmonary function than those infected with P.aeruginosa (Isles et al., 1984).

Although P.cepacia may become a problem in the future, at present mucoid P.aeruginosa is the predominant pathogen and cause of death in patients with CF (Høiby, 1982).

2.0 THE IMMUNE SYSTEM IN CYSTIC FIBROSIS

A considerable amount of work has been performed to determine whether or not an immunological defect is involved in CF. Such a defect could be either generalised or localised to the bronchial tree. However while CF patients are characteristically highly susceptible to respiratory tract bacterial infection, they are no more susceptible than normal individuals to infection at other sites, such as the skin, gut, urinary tract or central nervous system, and bacteraemia occurs rarely. This implies a defect in local rather than systemic defense mechanisms (Hodson, 1980). Both systems will be reviewed, to give a clear picture of the immunological state of the chronically infected CF patient.

2.1.0 The systemic humoral immune response

The total serum concentrations of IgG, IgA and IgM are normal to elevated in CF and tend to parallel the observed chronic infection (Schwartz, 1966; Wallwork et al., 1974). The majority of CF patients have high levels of serum antibody against bacterial outer membrane proteins and bacteria infecting their lungs (Speert et al., 1984). This may play a role in keeping the infection localised and explain the rarity of systemic infections observed in these patients (Høiby and Wilk, 1975). However, 20% of CF patients under the age of ten have hypogammaglobulinemia (Matthews et al., 1980). This condition is only found in patients with a low incidence of pulmonary infection as age-matched CF patients with chronic infection were shown to have high levels of serum antibody. It was thus suggested that an initial failure to recognise antigen when it crosses the gut or respiratory mucosa is followed by a hyperimmune response to the infecting organism (Matthews et al., 1980).

Serum precipitins are demonstrably increased after the emergence of mucoid P.aeruginosa (Doggett and Harrison, 1972) and their presence is closely associated with a poor prognosis (Høiby et al., 1975).

Antibody production has been investigated at the cellular level by a haemolytic plaque forming cell (PFC) assay (Götz et al., 1981). The ability of peripheral mononuclear cells obtained from patients with CF to form PFC upon stimulation with pokeweed mitogen was significantly decreased compared to healthy controls. However, there was no correlation between the level of serum IgG and the number of PFC cells, so this observation may be of no relevance in vivo (Götz et al., 1982). Other studies showed that the formation of spontaneous PFC that indicate in vivo activation of B cells was significantly higher in CF patients in good physical condition than controls, but depressed in patients with a bad prognosis, where suppression of antibody response may occur in vivo (Harper et al., 1980; Sorensen et al., 1983).

There is a high prevalence of autoantibodies in the serum and sputum of CF patients. IgG rheumatoid factor has been detected in 88% of patients and is not dependent on infection with P.aeruginosa (Schiøtz et al., 1979b). Antinuclear autoantibodies have also been detected and are probably associated with the tissue damage that accompanies prolonged respiratory infection (Høiby and Wilk, 1975).

2.1.1 Complement

There was no functional difference between CF patients and control subjects in any of the complement components, whole complement or C_3 inactivator, when a haemolytic complement assay was employed (Conover et al., 1973). Similarly, there were no differences observed between

C1q, C3,C4 and total haemolytic complement in patients infected with mucoid P.aeruginosa and those that had no infection (Schøitz et al., 1977). However using radial immunodiffusion C3 serum levels were found to be higher in CF than normal (Conover et al., 1973) or decreased in P.aeruginosa infected patients (Gotz and Lubec, 1978). However, these defects may represent secondary changes as total complement levels were similar in CF and normal.

2.1.2 Neutrophilis

There does not appear to be any inherent defect in neutrophils (PMN) from patients with CF (Hill et al., 1974). In fact they have been shown to be hyperfunctional when not in contact with autologous serum (Holland et al., 1981).

Chemotactic activity of PMN obtained from CF patients was found to be normal in one study that used the agarose plate method (Church et al., 1980), but significantly depressed in another study that used modified Boyden chambers (Zielinski et al., 1982). These contradictory results may be due to differences in the techniques employed for measuring chemotaxis and indicate that an inherent neutrophil defect is unlikely.

2.1.3 Lymphocytes

Absolute numbers of T and B lymphocytes in the blood increase with pulmonary infection, although the percentage of each cell type remains normal (Høiby and Mathieson, 1974). The ratio between helper (OKT4) and suppressor (OKT8) T cells has also been found to be normal (van Geffel et al., 1982; Wilson and Fudenberg, 1982), although increased

levels of suppressor T cells have been reported during chronic pulmonary infection in CF (Sorensen et al., 1980).

CF patients in good clinical condition have normal lymphocyte responses to P.aeruginosa (Sorensen et al., 1981a, 1981b). B cell transformation in response to poke weed mitogen or whole Staph.aureus was significantly impaired in CF patients in vitro and was not a result of increased suppression by T cells (Sorensen et al., 1983).

These lymphocyte abnormalities in CF probably arise as a result of pulmonary infection, as immunoglobulin levels are high and patients who are free from infection have normal responses. However any change in lymphocyte function may contribute to the establishment and pathogenicity of chronic pulmonary infection.

2.2 Pulmonary immune defense

The normal immune defense mechanisms of the lung have been extensively reviewed (Kaltreider, 1976; Green et al., 1977; Reynolds, 1978). This section will concentrate on immune function in CF and how it differs from that observed in the normal lung. Mucociliary transport is impaired in CF (Wood et al., 1975). However ciliary beat frequency in vitro is normal, suggesting that the abnormality is present in the mucus secretion (Rutland and Cole,1981).

2.2.1 Humoral immune response

The pulmonary humoral antibody response has been extensively studied in CF and a deficiency in total concentration of IgG, S-IgA or IgM has not been observed (review by Talamo et al., 1976). Precipitating antibodies to dietary proteins, allergens, body fluids

(McFarlane et al., 1975) and to the infecting organism are present (Schiotz, 1981).

Although the IgA antibody titre is higher in the sputum than the serum of chronically infected patients with CF, indicating local antibody production in the pulmonary tissue (Schiøtz et al., 1977), IgG is the predominant immunoglobulin in the secretion during infection due to passive transudation from the serum (Schiøtz, 1981).

Secretory IgA in the lung appears to be ineffective in CF against bacterial colonisation. However the only evidence for a structural abnormality of this immunoglobulin is the presence of free J chains in CF sputum (Wallwork et al., 1974) which may have no relevance in vivo. P.aeruginosa appears to be adequately opsonised as strains of bacteria isolated from CF sputa were shown to be coated with antibodies, predominantly of the IgA class (Hann and Holsclaw, 1976). However, the possibility that observations obtained from sputa may not reflect the in vivo situation in the lung, should be considered.

2.2.2 Pulmonary complement

Complement components have been demonstrated in the lung (Reynolds and Newball, 1974), but may be associated with inflammation and transudation from the serum as opposed to local production (Schiøtz et al., 1979a). However infection with P.aeruginosa has been shown to increase the synthesis of C_2 and C_4 by guinea-pig alveolar macrophages (Alpert et al., 1984) indicating that complement components can be produced in the lung. An increase in complement activation would enhance the inflammatory response and facilitate the recruitment of intravascular, cellular and humoral mediators.

2.2.3 Alveolar macrophages

One of the main functions of the alveolar macrophage is the phagocytosis and elimination of foreign particles including bacteria (Green and Kass, 1964). As mucociliary clearing mechanisms are impaired in the CF lung (Wood et al., 1975), the alveolar macrophage may be more important in bacterial clearance in CF than normal individuals.

Many studies have been performed to examine the possibility that the alveolar macrophage may be defective in CF. Results however have been contradictory and vary with the age of the patient and the technique used. However alveolar macrophages are obtained by bronchopulmonary lavage, so that the cells studied may not be the predominant cell-type or representative of the cell population within the lung.

Ultrastructural studies have shown that alveolar macrophages from CF patients are morphologically normal, although not heavily engaged in phagocytosis (Thomassen et al., 1980). Furthermore the phagocytic properties of cells isolated from CF adults were found to be normal in the presence of normal serum indicating the absence of any intrinsic defect of the cell. Alveolar macrophages obtained from CF patients were shown to contain intracellular bacteria that were capable of proliferation so that killing may be impaired in these cells (Hodson and Warner, 1984). Non-specific phagocytic activity as measured by yeast uptake was increased in alveolar macrophages from CF patients and was similar to that observed in control patients with respiratory infection (Cassino et al., 1980). Therefore the increase observed in phagocytic activity was a result of the infection and consequent activation of the macrophages and was not directly associated with CF. A study in children by the same workers showed a marked decrease in the "phagocytic

work" of alveolar macrophages from CF patients with pulmonary infection, while alveolar macrophages from non-CF patients with other forms of respiratory disease showed an increase in "phagocytic work" (Sordelli et al., 1982). These results were obtained by employing Michaelis Menten kinetics and were not dependent on the presence of autologous serum. The different methods employed to measure phagocytosis in these two studies may account for the apparent contradictory results.

It has been postulated that CF is the result of an inherent defect in the monocyte-macrophage (Wilson and Fudenberg, 1982). T lymphocytes and macrophages from CF homozygotes and obligate heterozygote carriers synthesize CF specific ciliary dyskinesia factors (CDF) that are not present in cultures of macrophages from healthy controls (Wilson and Bahm, 1980). These factors were first discovered in serum (*di Sant' Agnese and Davis, 1976; Wood et al., 1976) and have been shown to markedly increase the ciliary beat, cause excessive mucus production by goblet cells and the destruction of ciliary epithelium (Czegledy-Nagy and Sturgess, 1976; Wilson et al., 1977; Conover and Conod, 1978). Hyperproduction of both CDF and normal inflammatory mediators was shown to occur in CF macrophages (Wilson and Bahm, 1980). This metabolic defect appeared to be linked with an abnormality of CF alpha-2-macroglobulin, a regulating glycoprotein produced by macrophages (Mosher et al. 1977). These observations led Wilson and Fudenberg (1982) to postulate that the production of CDF by CF macrophages was responsible for the pathophysiology observed in CF patients and that the macrophage defect was genetically inherited. However this view is not widely accepted, as it is possible that the macrophage abnormalities are secondary to infection. Moreover macrophages from hetero-

(* reviewed by ...)

ozygote carriers produce CDF but this does not result in chronic obstructive lung disease (Wilson and Bahm 1980; Wilson et al., 1981)

2.3 Serum - Inhibitory factors

An early report by Biggar et al., (1971) showed that sera from CF patients supported normal phagocytosis by neutrophils but not by alveolar macrophages suggesting a dysfunction of the alveolar macrophages. Later phagocytosis by rabbit alveolar macrophages was found to be inhibited by fresh CF sera but not normal sera, while that of peripheral phagocytes was unaffected (Boxerbaum et al., 1973) and a serum opsonin defect for alveolar macrophage function in CF patients with frequent infections was discovered (Cole, 1979). CF serum has high opsonic activity for P.aeruginosa when neutrophils are studied (LeBlanc et al., 1982) but less than that of nonimmune sera or saline when alveolar macrophages are studied (Fick and Reynolds, 1980, 1983), indicating the presence of a serum inhibitory factor. Such an inhibitory factor had been indicated earlier as normal serum failed to overcome the inhibitory effect of CF serum on alveolar macrophages (Thomassen et al., 1979). The inhibitory factor was shown to specifically inhibit the phagocytosis of P.aeruginosa by alveolar macrophages, and it was suggested that the factor was a "blocking antibody" (Thomassen et al., 1979). Moreover the blocking antibody only occurs in CF patients with P.aeruginosa infection and was shown to have a defective Fc portion in the molecule (Fick and Reynolds, 1983). Thus the antibody coats the bacteria via its Fab portion, but attachment to the macrophage via the Fc portion is impaired. This molecular alteration of the Fc portion also occurs in the lung, where it is caused

by a metallo-proteinase, produced by P.aeruginosa, which enzymatically degrades immunoglobulin (Fick and Reynolds, 1983).

The blocking antibody has been shown to protect autologous P.aeruginosa strains from the bactericidal action of serum (Høiby and Olling, 1977; Thomassen and Demko, 1981) and is present as IgA in sputum (Schiller and Millard, 1983). The specificity of the inhibitory activity for alveolar macrophages has not been explained, but may be a result of an undetermined receptor difference between alveolar macrophages and monocytes (Thomassen et al., 1982).

A recent report has indicated that this blocking antibody is an IgG antibody against naturally occurring bactericidal IgM antibody specific for the strain of P.aeruginosa infecting the patient (Penketh et al., 1983). The presence of these blocking antibodies may partly explain the inability of the pulmonary defense mechanisms to clear P.aeruginosa from the lungs, and may further exacerbate respiratory pathology.

Human respiratory tract ciliary motility is unaffected by factors in CF serum (Rutland et al., 1983a, 1983b) that disrupt rabbit tracheal ciliary activity (Spock et al., 1967).

2.4 Immune complexes in Cystic Fibrosis

Patients chronically infected with P.aeruginosa have high levels of immune complexes in their serum (Schiøtz et al., 1977; Moss and Lewiston, 1980; Pitcher-Wilmott, 1982) and sputum (McFarlane et al., 1975). Circulating immune complexes are composed of P.aeruginosa antigens and serum precipitins, especially against LPS antigens (Moss and Lewiston, 1980; Berdischewsky et al., 1980).

The infecting strain of P.aeruginosa was shown to account for the antigenic specificity of the antibodies in immune complexes (Moss and Hsu, 1982), but not to be the sole antigenic stimulus (Manthei et al., 1982).

The presence of circulating immune complexes did not correlate with the initial severity of the lung disease or the clinical status of the patient (Pitcher-Wilmott et al., 1982). It was therefore postulated that immune complexes did not play a major role in pulmonary pathology but simply reflected the extent of lung tissue damage (Manthei et al., 1982). However this study was performed in children, where chronic infection may not be established. Circulating immune complexes are more common in advanced CF and occur at a higher frequency in chronically infected patients (Schiøtz et al., 1978).

Immune complexes affect histamine release and activate complement with a resulting increase in vascular permeability and infiltration of peripheral neutrophils and monocytes (Høiby and Schiøtz, 1982). Subsequently neutrophils are trapped and release lysosomal enzymes which cause tissue damage. Coagulation, kinin and fibrinolytic systems are also activated. Moreover immune complexes act as regulators of both cellular and humoral immune responses by virtue of their capacities to interact with antigen receptor-bearing lymphocytes and subpopulations of T and B cells, as well as with lymphocytes and macrophages that possess Fc and complement receptors (reviewed by Theofilopoulos and Dixon, 1979). Immune complexes may also suppress or enhance immune function, depending on the molar ratio of antigen to antibody, the epitope density of the complex and the class and affinity of antibody (review by Theofilopouos, 1980). For example enzymatic digestion of

immune complexes by macrophages results in Fc sub-fragments being released which non-specifically activate B cells (Morgan and Weigle, 1980).

Although immune complex formation is secondary to bacterial infection, it seems possible that their formation may lead to further tissue damage, by enhancing the immune response in hyperimmune patients. However, there is no direct evidence that immune complexes cause pulmonary damage in CF. The host may benefit as immune complexes are designed to eliminate and neutralise the antigens that are detrimental to the host.

2.5 Animal model systems for Cystic Fibrosis

The difficulties and limitations of working with humans have led to the search for a practical animal model to study CF. At present, no single animal model exists that exhibits all the aberrations present in the CF patient. However there are models that have been used to study particular aspects of CF and these will be briefly reviewed here.

A rat model of chronic respiratory infection has been described by Cash *et al.*,(1979). *P.aeruginosa* are enmeshed in agar beads and injected intratracheally into the lung. The agar beads protect the bacteria from the immune system that would otherwise eliminate them. Histologic examination of the infected lungs revealed lesions resembling those seen in the lung tissue of CF patients. This model is used in the investigation of the interactions between microbial virulence factors and host defense mechanisms. A similar model has been described in guinea-pigs (Blackwood and Pennington, 1981) and cats (Winnie *et al.*, 1982).

A recent study has shown that P.aeruginosa only required to be mixed with the agar beads to replicate and produce chronic respiratory infection in rats (Naccucchio et al.,1984). However other workers did not find that this procedure worked (Govan et al., unpublished results).

Rats and mice treated with high doses of reserpine have been used as a model for CF (Sordelli et al., 1978; Martinez et al., 1979). These animals have a disturbed secretory function of the respiratory tract and altered exocrine gland function. However the relevance of this model to CF is debatable as the reserpine affects the sympathetic nervous system and the animals become very ill as a result of the treatment.

The autosomal recessive cribriform degeneration mutation (cri) in DBA/2J mice was first described in 1972 by Green et al. Several studies since then have suggested that mice homozygous for the cri-gene might serve as an animal model for CF. Characteristics of the animals that resemble those of CF, include: increased salinity of the saliva, diminished ability to clear Staph.aureus from the lung and inflammation of the lung (Pivetta et al., 1977; Pivetta et al., 1981). It is intersting that kinetic studies have revealed decreased phagocytic capacity in alveolar macrophages and neutrophils from these animals (Cerquetti et al., 1983).

3.0 PSEUDOMONAS AERUGINOSA

P.aeruginosa is a gram negative bacillus which can survive in almost any reasonably moist environment. It can multiply in distilled water and antiseptic solutions, utilising the trace elements provided by dissolved carbon dioxide and organic matter.

The organism is an opportunistic pathogen and causes severe and debilitating infections in a variety of immunocompromised patients including those with CF. Conditions predisposing to P.aeruginosa infection include: natural immunologic deficiency (agammaglobulinemia); immunologic immaturity (premature infants); immunologic suppression (granulocytopenia); patients with extensive burns, underlying disease or undergoing respiratory procedures such as catheterisation or tracheostomy and patients with CF (Wood, 1976). During the last thirty years there has been a significant increase in the incidence of P.aeruginosa infection, particularly of nosocomial origin. The increased use of antibiotics, cytostatic and immunosuppressive drugs and more radical therapeutic and surgical practices may partly explain this increase (Frøland, 1981).

3.1 Products and virulence factors

P.aeruginosa produces many biologically active substances (reviewed by Woods and Iglewski, 1983). These include:

1) Lipo-polysaccharide, that has endotoxic activity (Greer and Milazzo, 1976), but is less toxic than other gram negative lipopolysaccharides and is not a primary virulence factor (Kostiala, 1980).

2) Exotoxin A, which inhibits protein synthesis by ribosylation in mammalian cells (Iglewski et al., 1977).

3) Several proteases and haemolysins that cause tissue damage (Liu et al., 1961; Kostiala, 1980).

4) A slime layer containing a leukotoxic glycolipoprotein (Lynn et al., 1977).

3.1.1 Effects on the immune system

A variety of studies on animals have shown that toxins produced by P.aeruginosa affect the immune system.

Supernatant from P.aeruginosa cultures, as well as living and killed bacteria have been shown to suppress cell mediated responses and cause prolongation of skin grafts in mice (Floersham et al., 1971). This immunosuppression was transferred by spleen cells and peritoneal macrophages from treated mice, and it was postulated that a product derived from P.aeruginosa altered macrophages and T lymphocyte activities by increasing the number of T suppressor cells (Petit et al., 1982). Some strains of P.aeruginosa produce an extracellular high molecular weight compound that inhibits the phagocytic and killing activities of rabbit neutrophils (Nonoyama et al., 1979; Kamimura et al., 1980). This substance is toxic and has been given the term neutrophil inhibitor. High concentrations of heat-killed P.aeruginosa are toxic to cultured neutrophils and monocytes (Garzelli et al., 1982). This is a result of the neutrophil inhibitor and other bacterial products.

Exotoxin A is toxic to human peripheral monocytes (Pollack and Anderson, 1978) and inhibits protein synthesis in mouse peritoneal macrophages (Iglewski et al., 1977). The exotoxin does not affect phagocytosis

but reduces the bactericidal activity of human neutrophils by inactivating complement (Weber et al., 1982). This product would appear to be an important virulence factor as purified preparations, injected intra-tracheally into rats produced histopathologic changes that resembled infection with the whole bacteria (Cash et al., 1983). A similar effect was also observed with proteases.

Alkaline protease and elastase have been shown to inhibit myeloperoxidase mediated chemiluminescence, a major antimicrobial system of human neutrophils (Kharazmi et al., 1984). Antibodies against the different toxins are produced in CF patients (Klinger et al., 1978) so that the toxins may be inactivated and their contribution to the pathogenesis of tissue damage in CF is at present uncertain.

3.1.2 Slime

The extracellular slime layer of P.aeruginosa is characteristic of the species and contains high molecular weight glycolipoprotein (GLP). In mice, the slime layer was shown to make a significant contribution to the pathogenesis of the bacteria, with GLP being the active component (Dimitracopoulos and Bartell, 1980).

The slime acts as a protective antigen and will cause leukopenia and death when injected intraperitoneally into mice (Dimitracopoulos et al., 1974; Lynn et al., 1977). Neutropenia is induced by the GLP, which binds to the neutrophils to form a complex that localises in the liver and is subsequently removed from the circulation. In animals that are immune to the organism, antibody binds to the GLP and inhibits its attachment to the neutrophils (Lynn et al., 1977).

Slime is antiphagocytic (Sensakovic and Bartell, 1974) and impairs

the *in vitro* motility, endocytosis and phagosome formation of human neutrophils (Laharrague *et al.*, 1984). The carbohydrate moiety of the GLP molecule is responsible for its antigenic specificity and inhibition of phagocytosis, whereas the lipid moiety is associated with the leucopenic and lethal effects (Sensakovic and Bartell, 1975). This profound impairment of neutrophil function, without alteration of viability may contribute to the virulence of the organism.

3.2 Mucoid Pseudomonas aeruginosa

Clinical and bacteriological studies of patients with CF have indicated that initial colonisation or infection occurs with a typical non-mucoid strain of *P.aeruginosa*. Once colonized the gradual emergence of the mucoid form is almost inevitable and is associated with clinical deterioration and a poor prognosis (Doggett and Harrison, 1972; Høiby *et al.*, 1975). The mucoid form is a mutant of the non-mucoid strain and produces large quantities of an alginate-like exopolysaccaride *in vivo* and on broth culture *in vitro*. The properties of alginate will be reviewed in a later section. Despite the wide range and variety of infection due to *P.aeruginosa*, the mucoid form is seldom encountered except in patients with CF (Doggett, 1969). Moreover the association between mucoid *P.aeruginosa* and CF became so characteristic that it was suggested to be diagnostic of CF (Reynolds *et al.*, 1976). By the time of post mortem examination, Kulczycki *et al.*, (1978) observed that almost 100% of patients who died of pulmonary tract complications had cultures positive for *P.aeruginosa*, 90% of which were the mucoid form. Mucoid *P.aeruginosa* are infrequently isolated from non CF patients where they are either associated with chronic lung infection (Høiby, 1975) or

obstruction of the airways (Clarke et al., 1981).

3.2.1 The role of mucoid P.aeruginosa in cystic fibrosis

The association between mucoid P.aeruginosa and CF has not yet been adequately explained. The factors that may be involved in the emergence of the mucoid form in CF will be discussed here.

The fact that the CF lung is already obstructed by viscid mucus and that mucociliary clearance is impaired (Rutland and Cole, 1981) may be an important factor in the emergence of mucoid P.aeruginosa and mucus excess may help to keep the infection localised. Most P.aeruginosa isolates from CF patients are capable of mutation to the mucoid form if the correct medium is used (Lam et al., 1980). However the environment of the lung does not switch on mutation but acts as a selective environment for the establishment of the mucoid form (Govan et al., 1979). In vitro, although the mutation is easily observed, the environment of most culture media favours the growth of non-mucoid revertants, so that mucoidy may be lost in culture but not in the CF lung. Further evidence that the CF lung may be conducive for mucoid variants of bacteria, was obtained by the isolation of mucoid strains of E.coli from CF sputa (Macone et al.,1981).

Levels of calcium are elevated in CF and concentrations as high as 0.002 mg ml^{-1} have been documented in CF respiratory secretions (Kilbourn, 1978). In the absence of suitable electrolytes Pseudomonas alginate is produced as an extracellular slime and is easily removed by washing. In the presence of calcium ions, however, the cells become embedded in a protective matrix or microcolony surrounded by their own alginate. Thus these elevated calcium levels may provide a positive selective

pressure for the emergence of the mucoid phenotype.

The continuous use of antibiotics may contribute to the persistence of P.aeruginosa and the appearance of the mucoid strains (Kulczyki et al., 1978), however this seems unlikely to be the sole explanation as mucoid P.aeruginosa have been isolated from patients with no history of antimicrobial therapy (Doggett and Harrison, 1969). In early studies mucoid variants were reported to be more resistant in vitro to B-lactam and aminoglycoside antibiotics than related non-mucoid strains (Govan and Fyfe, 1978). Later studies showed that some mucoid strains were extremely sensitive to antibiotics such as carbenicillin (Govan et al., 1983). This apparent contradictory finding is explained by the demonstration of an independent mutation responsible for hypersensitivity to antibiotics, which overcomes the slight enhancement of resistance due to the mucoid layer (Govan et al., 1983; Fyfe and Govan, 1984). The fact that mucoid strains are hypersensitive to antibiotics yet persist after antibiotic therapy, may be a result of the microcolony mode of growth as the alginate matrix may protect the bacteria from the antibiotic. Alternatively antibiotic hypersensitivity in vivo may be an adaptation to the environment, by providing enhanced permeability and uptake of essential nutrients through an otherwise impermeable alginate matrix (Govan, 1983).

The formation of microcolonies surrounded by alginate, renders the bacteria resistant to immune clearance (Costerton et al., 1983). Mucoid P.aeruginosa are also resistant to opsonisation by antibody directed against the immunotype specific antigen (Baltimore and Shedd, 1983). However, even if opsonisation had taken place, a large microcolony, surrounded by alginate would probably resist phagocytosis by macrophages.

A microcolony mode of growth would also protect the bacteria from the bactericidal action of serum. This would explain the *in vitro* observation that mucoid strains are more serum sensitive when compared with non-mucoid forms (Thomassen and Demko, 1981).

Tissue damage due to pseudomonas toxins would also be enhanced by microcolony formation since the concentration of the bacteria within a matrix would act to localise toxins and reduce the diluting effect of body fluids.

Alginate production and hypersensitivity to antibiotics are both associated with advanced chronic infection and are due to bacterial mutations of non-mucoid *P.aeruginosa* that initially colonised the patient. Therefore CF patients may be more susceptible to initial colonisation with *P.aeruginosa* than normal. CF patients have raised levels of salivary proteases and *P.aeruginosa* were shown to adhere to a greater extent to buccal cells from CF patients than to similar cells from control subjects (Woods *et al.*, 1980). Previous studies (Johanson *et al.*, 1979) had shown that control cells behaved similarly to CF cells if their normal coating of fibronectin was reduced by treatment with the proteolytic enzyme, trypsin. Therefore the raised levels of proteases reduce the level of fibronectin that leads to increased binding of *P.aeruginosa* (Woods *et al.*, 1983). Non-mucoid bacteria adhered better than mucoid bacteria to buccal cells. Adhesion to the buccal cells was mediated by hair-like pili on the bacterial surface which was masked by the presence of alginate. However in the lower respiratory tract, only mucoid *P.aeruginosa* adhered in significant numbers (Baker and Marcus, 1982). Mucoid bacteria adhered in the form of microcolonies rather than as individual cells. Mucoid bacteria are

metabolically less efficient (Mian, 1978) yet supersede non-mucoid bacteria in CF. The emergence and persistance of mucoid P.aeruginosa in CF is probably a result of many inter-related factors, although in large part may be a result of the microcolony mode of growth and protection of the bacteria within an alginate matrix.

3.2.2 Heterogeneity

Mucoid P.aeruginosa are often thought to be a homogenous class of species possessing basic common properties. However physiological and genetic studies have revealed heterogeneity within this form of the species (Irvin et al., 1981; Govan et al., 1983). A heterogenous population of mucoid variants are often found within sputum taken from individual CF patients and this is not explained by simultaneous infection with more than one strain. The heterogeneity may involve forms that are indistinguishable in colonial appearance, yet show differences in the synthesis and chemical structure of alginate and hundred fold differences in susceptibility to B-lactam antibiotics (Govan et al., 1983). This heterogeneity serves to illustrate the complexity of the natural history of P.aeruginosa chronic pulmonary infection that occurs in CF.

3.3 Pseudomonas alginate

The extracellular exopolysaccharide produced by mucoid variants of P.aeruginosa is quite distinct from the slime material characteristically secreted by non-mucoid strains (Doggett et al., 1964) and referred to earlier. This material has been referred to as alginate, glycocalyx, mucoid material or slime. Confusion has often arisen in the literature, as the term slime has been used to describe extracellular

substance from mucoid (Evans and Linker, 1973) and from non-mucoid strains (Bartell et al., 1970). The term alginate has been used in this thesis to distinguish the material produced by mucoid forms of P. aeruginosa from the characteristic slime of the species.

Alginate is a polyuronide composed of D-mannuronic acid and L-guluronic acid linked by O-acetyl groups (Linker and Jones, 1966). The structure is similar to alginate produced by the bacterial species Azotobacter vinelandii and by marine algae, except the algal alginate is non-acelylated. The relative amounts of the two monomers and their physiological properties are dependent on the calcium ions present in the nutrients (Doggett et al., 1971; Govan et al., 1983).

3.3.1 Physical properties

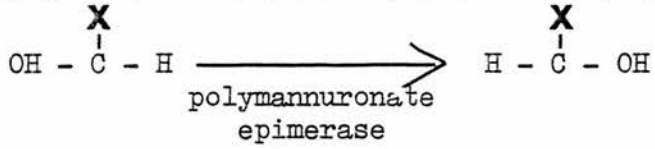
Pseudomonas alginate has a molecular weight of 10,000 daltons or more (Speert et al.,1984) and is highly viscous in nature. The polysaccharide is produced extracellularly in the form of a loosely associated peripheral capsule as opposed to a distinct, cell-bound bacterial capsule (Costerton et al., 1981; Marty et al., 1981). The degree of viscosity or rheological properties are significantly influenced by divalent cations which link the negatively charged carboxylic acid moieties and are increased in the presence of calcium ions (Pier et al., 1983).

In contrast to Pseudomonas slime (Liu, 1979), the purified alginate is nontoxic when injected intraperitoneally into mice (J.R.W. Govan, personal communication).

3.3.2 Biosynthesis

Pseudomonas alginate is synthesised initially as a homopolymer of

mannuronic acid, subsequently a proportion of the mannuronic acid residues are converted to guluronic acid by a C5 epimerisation (rotation around the C bond) (Sutherland, 1977; Fyfe and Govan, 1983).



The acetyl groups are added from acetyl CoA before the polymer is secreted into the medium. Portions of the polymer containing O-acetyl mannuronic acid may then be protected from epimerisation. The degree of acetylation of the alginate is proportional to the viscosity of the polymer (Piggott, 1979). Individual mucoid strains differ considerably in the amount of alginate produced, the ratio of the two monomers, the degree of acetylation and the optimal conditions for synthesis of the alginate (Evans and Linker, 1973).

3.3.3 Effects on the immune system

As mucoid producing strains of P.aeruginosa are seldom eradicated, once established in the CF lung, it would seem that the production of alginate may confer a selective advantage on the bacteria. One advantage might be that the alginate protects the bacteria from the hosts immune responses and thus plays an important role in the establishment and chronicity of the infection. The advantage conferred by the micro-colony mode of growth in this way has previously been discussed.

A report by Schwartzmann and Boring (1971) showed that Pseudomonas alginate inhibited the phagocytosis of E.coli, Staph.aureus and P.aeruginosa. However no distinction was made between phagocytosis and intracellular killing in the assay performed, as cells were lysed and colony counts performed to determine the percentage of killed bacteria

in treated and untreated cells. Mucoid forms were shown to resist phagocytosis by cultured rat and guinea-pig alveolar macrophages (Ruhen et al., 1980). Cell free alginate was also shown to inhibit phagocytosis of the non-mucoid variant when added with the bacteria. However this was in the form of a preliminary report and did not include details of the procedures employed.

Mucoid P.aeruginosa strains that produced moderate amounts of alginate were shown to be killed by human neutrophils, whereas high producing strains were ineffectively killed (Roe and Jones, 1974), indicating that alginate protected the bacteria from microbicidal mechanisms. A later study reported that mucoid strains were ineffectively opsonised with antibody specific for the non-mucoid revertant (Baltimore and Mitchell, 1980). It was concluded from these observations that the immunodeterminant for opsonic antibody was blocked in the mucoid form by alginate. However in the same study a crude alginate extract had no effect on the opsonisation of the non-mucoid revertant, when added to a mixture of antibody and bacteria. This latter result appears to contradict the former and may be a result of the alginate purification technique.

Alginate has been shown to inactivate the classical complement pathway by primary inactivation of C2, which results in poor opsonic activity and impaired killing of Staph.aureus by human neutrophils (Speert et al., 1981).

The contradictory results that are sometimes obtained in different studies may be explained by differences in techniques. For example, after washing, mucoid P.aeruginosa are no longer mucoid, as during centrifugation most of the alginate remains in the culture supernate

(Personal Observation). In the course of a short experiment there would be little prospect for alginate synthesis *in vivo* (Govan, 1983). There was no difference observed in the pulmonary clearance of mucoid and non-mucoid *P.aeruginosa* inoculated intratracheally into guinea-pigs, when the organisms were washed (Blackwood and Pennington, 1981). Similarly no differences were observed in the opsonic requirements and phagocytosis of mucoid and non-mucoid strains when studied with an assay using radio-labelled bacteria that involved washing the bacteria (Meshulam *et al.*, 1982). However increased pulmonary survival of mucoid *P.aeruginosa* was observed in rats killed three and six hours after intra-tracheal inoculation with unwashed bacteria (Govan *et al.*, 1983). This effect did not occur when the bacteria were washed prior to inoculation.

Thus the alginate may render mucoid *P.aeruginosa* less susceptible than non-mucoid forms to normal pulmonary bactericidal mechanisms. This action together with mechanical obstruction, microcolony mode of growth, and rheological properties in the presence of calcium may partly explain the persistance of the mucoid form and its association with a poor prognosis. Further actions of the alginate on pulmonary immune defenses will be described in this thesis.

3.3.4 Immunogenicity

Until recently, it was generally accepted that alginate was non-immunogenic, as humoral or cellular responses against the alginate were not observed (Høiby *et al.*, 1975; Lam *et al.*, 1980). However in 1981, Macone *et al.* reported that rabbits immunised with mucoid *P.aeruginosa* produced high titres of antibody, specific for the alginate. The

activity was absorbed with mucoid, but not non-mucoid organisms and the immune serum reacted specifically with purified alginate. One factor that possibly interferred with the search for an anti-alginate antibody in CF patients, may have been the lack of a reliable and sensitive procedure for its detection. This was overcome by Bryan et al., (1983) and later by Speert et al., (1984) by the development of an enzyme-linked immunosorbent assay (ELISA), with alginate as the antigen. This technique allowed low levels of anti-alginate antibody to be detected in the sera of several patients with CF, demonstrating that the alginate was immunogenic. At the same time another group of workers discovered anti-alginate antibody by performing a haemagglutination assay (Pier et al., 1983). The mucoid antigen from one strain was found to be composed predominantly of a single serologic epitope, that was shared with forty other chemical isolates. In addition to the common epitope, alginate from some strains also possessed a serotype-specific determinant. CF patients colonised with P.aeruginosa had higher antibody titres than patients who were not colonised and antibody was absent in healthy controls (Speert et al., 1984). The antiserum was shown to cross-react with seaweed alginate, but not other bacterial polysaccharides.

The anti-alginate antibody produced by CF patients appears to be an ineffective opsonin, as mucoid strains persist and are rarely eradicated. The reason for this could be that the antibody does not reach the lung or is "mopped-up" by an excess of alginate (Lam et al., 1980). Alternatively opsonisation could take place but the sheer size of the Pseudomonas microcolony enclosed within an alginate gel frustrates phagocytosis (Govan, 1983). The formation of alginate antibody may even be harmful, if it participates in an arthus reaction in the

lung (Zavala et al., 1975) or forms part of the immune complexes that occur in CF sera and sputum (Manthei et al., 1982).

3.4 Pseudomonas aeruginosa vaccines

Although several P.aeruginosa vaccines have been developed, it would seem unlikely that active immunisation would be of use to those CF patients who already have chronic pulmonary infection and high antibody titres in the serum and sputum. Such patients might be considered to be already hyperimmune. Patients with CF immunised with a multivalent P.aeruginosa LPS vaccine were shown to have increased serum antibody titres (Pennington et al., 1975). However the clinical course was unchanged and P.aeruginosa was not eradicated from the sputum. Patients immunised intranasally with the same vaccine, showed a slight increase in serum antibody titres, although sputum levels remained the same. On the other hand, intranasal application of the vaccine to rabbits led to a rise in the antibody titre in the lung and serum (Wood et al., 1983). Another study showed that serum antibodies against envelope proteins did not protect CF patients from further pulmonary infection with P.aeruginosa (Fernandes et al., 1981).

Prophylactic immunisation against P.aeruginosa may be effective in preventing initial colonisation, the emergence of mucoid P.aeruginosa and the establishment of chronic infection. Vaccination of mice with a high molecular weight polysaccharide from mucoid P.aeruginosa, protected them against a challenge with the organisms (Pier et al., 1978). Similarly a polyvalent cell wall extract protected guinea-pigs from pneumonia, following challenge with P.aeruginosa (Pennington and Miller, 1979), and the polyvalent vaccine (PEV-01) protected rats against an

intra-tracheal challenge of P.aeruginosa in agarose beads (Klinger et al., 1983). Protection in the rats may have been a result of immunisation against toxic exoproducts, as although lung inflammation was minimal, the bacterial numbers in the lungs were no different in treated and untreated rats. At present, no study on prophylactic immunisation in CF patients has been reported.

Vaccines against P.aeruginosa have been shown to be effective in non-pulmonary infections in animals and man. A ribosomal vaccine produced active immunity in mice against intra-peritoneal injection of P.aeruginosa (Lieberman et al., 1979). A study in India reported that a polyvalent vaccine decreased the mortality due to systemic Paeruginosa infection (Jones et al., 1979) and a high molecular weight polysaccharide from slime has been shown to be an effective vaccine against burn infections (Pollack et al., 1984). Rabbit antisera produced against the polysaccharide was shown to passively protect mice from infection.

As P.aeruginosa antibody produced by chronically infected CF patients may be functionally inefficient (Fick et al., 1984), qualitative replacement of specific IgG anti-Pseudomonas antibody may be more appropriate than prophylatic vaccination. From the previous discussion it can be seen that many factors are likely to be involved in the predisposition of CF patients to lung infection with P.aeruginosa, so that prophylatic vaccination may be of little use.

4.0 MACROPHAGES

Macrophages are derived from peripheral blood monocytes, reside in the tissues and are part of the mononuclear phagocyte system (van Furth, 1982). The maturation of monocytes into macrophages occurs without cell division, but with a more than three fold increase in cell volume (Gordon and Cohn, 1973). Monocytes are a mobile, functionally diverse group of cells that may be recruited to tissues and stimulated to a high degree of metabolic activity. Monocytes are chemotactically attracted to an infected focus, where they may differentiate into macrophages. Many substances are chemotactic for monocytes and they include bacteria and their products, immune complexes, complement components, kallekrein and lymphokines released by activated T lymphocytes (Wilkinson, 1976). Macrophages are also able to accumulate and remain at an infected focus under the influence of a migration inhibition factor (MIF) released by T lymphocytes (Racklin et al., 1980). Macrophages have a marked capacity for endocytosis and the ability to adhere tenaciously to glass or plastic surfaces. They also secrete various enzymes, including lysozyme, complement components, prostaglandins and other substances that may mediate or modulate tissue damage and repair (Hopper et al., 1979). Macrophages are also very important effector cells in cell mediated immunity and act as accessory cells for many T lymphocyte functions. Other functions ascribed to macrophages include numerous homeostatic functions unrelated to host defence, such as removal of senescent red blood cells, turnover of lung surfactant and bone remodelling and resorption (Douglas, 1980; Silverstein, 1982). Macrophage heterogeneity may be detected between populations from different sites or between macrophages within a population obtained from a particular

site (reviewed by Dougherty and McBride, 1984). There are many general reviews concerning the macrophage (Hopper et al., 1979; Lasser, 1983; van Furth, 1983). This discussion will concentrate on the alveolar macrophage and emphasize the ways in which these macrophages differ from other macrophage populations, especially peritoneal macrophages.

5.0 ALVEOLAR MACROPHAGES

The alveolar macrophage is the resident mononuclear phagocyte and the most prominant inflammatory and immune effector cell in the lung (Crapo et al., 1982). Bronchoalveolar lavage fluid taken from a normal adult contains 93% ± 5% macrophages, 7% ± 1% lymphocytes and less than 1% neutrophils, eosinophils or basophils (Hunninghake et al., 1979). Their main function is in the primary defence against inhaled particulate matter (Green and Kass, 1964) so that the lung is maintained as a clean and sterile surface suitable for gas exhange. The phagocytes function primarily on alveolar surfaces rather than within tissues, and move around on the alveolar surface within the liquid lining layer (Burri, 1983). Alveolar macrophages display most of the typical macrophage traits but also exhibit certain unique characteristics that are discussed below.

5.1 Origin and kinetics

Alveolar macrophages are derived from progenitors in the bone marrow. A bone marrow monoblast divides to give rise to two promonocytes which divide once to give rise to two monocytes. The monocytes leave the bone marrow and enter the circulation where they remain (in man the half-life is seventy-one hours) unless they migrate into the tissues where they differentiate into macrophages (van Furth, 1981).

In animals given lethal whole-body irradiation, followed by an infusion of donor bone marrow cells the alveolar macrophage population has been shown to be substantially replaced with cells of donor origin (Blussé van oud Alblas and van Furth, 1979). Human alveolar macrophages

have also been shown to be derived from the bone marrow (Thomas et al., 1976). Monocytes differentiate into morphologically typical alveolar macrophages within a few hours of entering the alveolar air spaces (Blusse van oud Alblas et al., 1983a). These workers preclude the existence of an interstial pool of dividing precursor cells. However a dual mechanism of monocyte differentiation into macrophages has been postulated (Adamson and Bowden, 1980; Bowden and Adamson, 1980). The monocytes either cross the interstitium without dividing and differentiate into alveolar macrophages or become interstitial cells that subsequently divide and migrate to the alveoli to become macrophages. Thus the lung interstitium may be an intermediate environment between the blood and alveolar space. Genetic markers have shown that blood monocytes do populate the interstitial and alveolar compartments of the lung (Johnson et al., 1980b). Therefore the monocytes may develop functional and metabolic features of the alveolar macrophage in the interstitium, prior to their efflux into the alveoli. Using Fc receptor avidity as a marker, alveolar macrophages have been shown to be more stimulated than peripheral blood monocytes (Holt et al., 1982).

Alveolar macrophages are continually renewed to balance the loss of cells through the airways and lymphatic channels (Bertalanffy, 1964). However, whether the population is sustained wholly by monocyte efflux into the lung or by local division of resident alveolar macrophages is controversial.

Rodent alveolar macrophages have the capacity to proliferate in vivo (Naum, 1975) and nitrous oxide is a potent stimulator of alveolar macrophage proliferation (Evans et al., 1973). Similarly hamster alveolar macrophages

will proliferate and form colonies in soft gel agar (Lin et al., 1975). Human alveolar macrophages are also capable of replication (Golde et al., 1974). However the in vivo importance of this capacity for replication is uncertain. In mice it has been demonstrated that 70% of the alveolar macrophage population is supplied by monocyte influx into the lung and 30% by local division of immature monocytes originating from the bone marrow (Blusse van oud Alblas et al., 1983b). These workers claim that the maintenance of the alveolar macrophage population is dependent on the influx of circulating monocytes and only to a minor degree on local division. They also postulate that the increase in the alveolar macrophage population during local inflammation is a result of an increased influx of peripheral monocytes as opposed to an increase in division of the resident population (Blusse van oud Alblas et al., 1983a, van Furth and Blusse van oud Alblas, 1983). However no evidence is presented to show that the dividing population of alveolar macrophages are solely new monocytes recently recruited into the lung. Although these workers now admit that a dividing alveolar macrophage population exists, they claim it is unimportant in sustaining the population. They argue that other workers who claim that the dividing population may be important have not used adequate techniques for identifying the alveolar macrophage from other pulmonary macrophages (van Furth and Blussé van oud Alblas, 1982).

Contrary to these views is the proposal that alveolar macrophages are a self-sustaining population (Coggle and Tarling, 1983). These workers showed that the labelling pattern of alveolar macrophages was only altered after lung irradiation and that it did not change after bone marrow irradiation. Macrophage numbers remained constant despite

a severe depression in the numbers of peripheral monocytes. Flash labelling studies also revealed that 30% of the resident alveolar macrophage population were involved in DNA synthesis. Previously it had been reported that patients with acute leukemia, maintained their alveolar macrophage population despite prolonged periods of monocytopernia (Golde et al., 1974). Therefore it would appear that under certain conditions the alveolar macrophage population can be self-sustaining.

Cellular transformation can be modelled in vitro. Human monocytes and alveolar macrophages have different enzyme patterns, however after culture, monocytes have been shown to display the alveolar macrophage pattern (Radzun et al., 1983).

Thus whilst studies definitely show that that alveolar macrophage is originally derived from the blood monocyte, controversy remains over the more important mechanism (monocyte influx or local cell division) involved in sustaining the alveolar macrophage population, once it is established. The macrophages apparently have the capacity to divide, but whether this is limited to a population of newly differentiated monocytes is open to question. The system may be adaptable, so that under certain conditions, for example, monocytopenia, local macrophage division would increase as opposed to monocyte influx to maintain the alveolar macrophage population.

5.2 Characteristics

Alveolar macrophages differ from other macrophages in structural, biochemical and functional properties (Sorokin, 1979; Hocking and Golde 1979; Hunninghake et al., 1979). Monoclonal antibodies have

also indicated antigenic differences between alveolar and peritoneal macrophages (Rumpold et al., 1981).

Alveolar macrophages vary in size from fifteen to fifty microns in diameter and have a nucleus to cytoplasmic ratio of approximately one to three. When attached to glass they have an elongated and flat or spherical appearance with a raised central area. They attach readily to glass and this is accomplished by lamellipodia and filapodia (Hocking and Golde, 1979). They contain large numbers of mitochondria and an increased content of mitochondrial enzymes, compared to other macrophages. Similarly they have more numerous and larger cytoplasmic inclusions containing lysosomal hydrolytic enzymes, and produce substantially higher amounts of acid phosphatase, B-glucuronidase (Hearst et al., 1980) and prostaglandins (Morley et al., 1979).

5.3 Oxidative Metabolism

The aerobic environment of the alveolus has led the alveolar macrophage to develop special metabolic adaptations (Simon et al.,1977). At rest they have a higher 14C-1-glucose metabolism (Paper Master-Bender et al., 1980) and phosphatase activity (Hearst et al., 1980) than peritoneal macrophages. The respiratory rate of the alveolar macrophage is greater than that of any other mammalian phagocytic cell. For phagocytosis to occur, a higher level of energy supplied through oxidative phosphoylation is required than for comparable numbers of peritoneal macrophages (Karnovsky et al., 1970). Resident alveolar macrophages show high levels of glucose and oxygen consumption, with only slight changes during phagocytosis, when production of toxic metabolites is low, compared to that of peritoneal macrophages (Oren et al., 1963).

Incubation of alveolar macrophages under anaerobic conditions will inhibit phagocytosis and particle ingestion is decreased in the presence of inhibitors of glycolysis or oxidative metabolism (Ouchi et al., 1965; Cohen and Chovaniec, 1978). In contrast to other phagocytes, alveolar macrophages depend on both oxidative and glycolytic metabolism for phagocytosis to occur.

The activities of oxygen metabolising enzymes of either peritoneal or alveolar macrophages can vary depending on whether they are maintained in an aerobic or anaerobic environment. For example, when alveolar macrophages are incubated in vitro under anaerobic conditions, they exhibit an enzymatic pattern similar to that of peritoneal macrophages (Simon et al., 1977).

Alveolar macrophages usually depend on an oxygen tension that is greater than 25 mm Hg for normal phagocytosis (Cohen and Cline, 1971). The differences observed between alveolar and peritoneal macrophage function may therefore sometimes be a result of the culture technique employed. Alveolar macrophages may function optimally when cultured on coverslips, where oxygen tension is high, whereas peritoneal macrophages may be more suitably studied in suspension under anaerobic conditions (Hearst et al., 1980).

Unlike peritoneal macrophages, the alveolar macrophage has low amounts of myelo-peroxidase mediated antimicrobial activity. However high concentrations of catalase are also observed after ingestion and it is this enzyme that functions primarily as a peroxidase in these cells (Stossel et al., 1972; Mauel, 1982).

5.4 Role in immunity

The principal role of the alveolar macrophage is in the primary defense of the lung against inhaled bacteria and inert particles (Green and Kass, 1964; Goldstein *et al.*, 1974). They also participate in specific immunological reactions, such as antigen presentation and cell mediated immunity and interact with other components of the immune system acting as accessory cells (Nichols, 1980).

5.4.1 Surveillance and phagocytosis

Alveolar macrophages are dispersed throughout the alveoli, so that they intercept bacteria within minutes of their entry into the lung. Bacteria are then ingested, inactivated and degraded in two to four hours (Jakab, 1981). Rats infected intratracheally with *Staph.aureus* cleared 76.9% of the bacteria in five hours and 87.1% were found to be intracellular within five hours (Goldstein *et al.*, 1974). The efficiency of this phagocytic mechanism maintains the sterility of the lung, under normal conditions, despite the constant influx of bacteria and other particles. However during pulmonary infection, the role of the alveolar macrophage is augmented by an influx of blood monocytes and neutrophils and antibody production.

Human alveolar macrophages *in vitro* have been shown to exhibit a slight decrease in chemotaxis, phagocytosis and microbial killing compared to similar numbers of peripheral blood monocytes (Territo and Golde, 1979). A later study showed that the ability to phagocytose *Staph.aureus* and *E.coli* was greater for human alveolar macrophages than blood monocytes, although the bactericidal activity of the latter was greater (Hoidal *et al.*, 1981). In Swiss mice alveolar macrophages

have a greater phagocytic capacity than peritoneal macrophages (Hearst, 1980), while in Sprague-Dawley rats the reverse is observed (Personal observation).

5.4.2. General immune functions

Alveolar macrophages are cytotoxic for virus-infected cells (Stott et al., 1975) produce interferon (Acton and Myrvik, 1966) and are important in antitumour immunity (Sone and Fidler, 1980).

They produce a colony stimulating factor that induces granulocyte and monocyte stem cell replication in vitro (Golde et al., 1972) release factors chemotactic for monocytes and neutrophils (Hunninghake et al., 1979) and mediate the amplification of neutrophil bactericidal mechanisms (Pennington et al., 1983). Thus the alveolar macrophage has the potential to stimulate the production of neutrophils and monocytes by the bone marrow, induce their migration to the lung and sites of infection and subsequently amplify their immune responses. The alveolar macrophage is clearly an important effector cell during a pulmonary infection and may initiate the inflammatory response. Alveolar macrophages do not form spontaneous rosettes with untreated sheep erythrocytes, but they do form high numbers of rosettes with Candida (Hearst et al., 1980). They are efficient at cleaning yeast from the lung, due to the presence of a lectin-like receptor specific for mannan phosphate (Sung et al., 1983). These cells are unique among macrophages in their ability to inhibit the expression of natural killer (NK) cell activity (Bordignon et al., 1982). NK activity is low in the lung and may serve to protect the pulmonary tissue from damage.

5.5 Accessory cell function

Alveolar macrophages are not purely scavenger or surveillance cells, but act as accessory cells in the induction and expression of humoral and cell-mediated immune responses (Unanue, 1978).

As they are located on the surface of the alveoli, alveolar macrophages are in an ideal position to recognise and bind antigens and subsequently present them to T lymphocytes. It has been shown that antigen inoculated intratracheally into guinea-pigs can induce a primary immune response and selectively recruit specific T lymphocytes to the lung (Lyons and Lipscomb, 1983). Alveolar macrophages are capable of interacting with antigen in a manner analogous to that of other macrophages. However they appear to be relatively inefficient in presenting antigen for the initiation of an immune response (Ullrich and Herscowitz, 1980). There are marked species differences in the accessory immune function of alveolar macrophages which may be related to the number of Ia positive cells in the population.

5.5.1 Animal studies

Murine alveolar macrophages have been shown to be capable of supporting PHA induced lymphoproliferation (Holt, 1980). Another study found that murine alveolar macrophages inhibited the splenocyte response to Con.A as a result of prostaglandin release that caused the cytostasis of lymphocytes (Sestini *et al.*, 1982). Other observations are contradictory, for on the one hand murine alveolar macrophages did not support proliferation of lymphocytes to soluble antigen (Ullrich and Herscowitz, 1980) while on the other they were shown to be as potent as spleen cells at presenting alloantigen or soluble

protein antigen to cloned T cell hybridomas (Daughety et al., 1983), but less effective accessory cells than similar numbers of peritoneal macrophages (Weinburg and Unanue, 1981; Shellito et al., 1983). The latter study also showed that interleukin 1 production was lower and that fewer lymphocytes were bound by alveolar macrophages than peritoneal macrophages. Differences between the studies may be related to the different strains of mice used in each experiment. 50% of murine alveolar macrophages are Ia positive and proliferation of primed T cells propagated in vitro is induced by cells that possess functional products of IA and IE subregions (Daughety et al., 1983). When antigen presentation assays are carried out with murine alveolar macrophages they can be shown to be dose-dependent, antigen specific and genetically restricted (Weinburg and Unanue, 1981).

Rat alveolar macrophages suppress mitogen-stimulated lymphoproliferation, are cytostatic and only support low levels of T cell activation (Holt, 1979a, 1980; Kaltreider, 1982). The percentage of Ia positive alveolar macrophages in the rat is low, 10% in Sprague-Dawley (Personal observation) and may account for the observed ineffective accessory cell activity of these cells. A study on sub-populations of rat alveolar macrophages, separated according to density, showed that cells from the lowest and highest density fractions had a minimal effect on mitogenesis, while cells from the intermediate density fractions caused suppression (Shellito and Kaltreider, 1984). Therefore these cells exhibit differences in their ability to act as accessory cells, depending on the degree of cell maturation.

Guinea-pig alveolar macrophages were shown to support PHA induced

lymphoproliferation and to present soluble antigen, with a response greater than that observed with peritoneal macrophages (Holt and Batty, 1980; Kaltreider, 1982). These cells fail to suppress lymphoproliferation even at high ratios. Their efficiency as accessory cells may be a result of the high percentage (80%) of Ia positive cells in the population (Lipscombe et al., 1981).

Studies on rabbit alveolar macrophages on the other hand have been contradictory and confusing. The macrophages have been shown to inhibit PHA induced lymphoproliferation (Holt, 1980). However in one study low alveolar macrophage to lymphocyte ratios(1:100) were shown to enhance the lymphocyte response to Con.A and sheep erythrocytes, while high ratios (1:1) caused suppression of the response (Pennline and Herscowitz, 1981). In another study high alveolar macrophage to lymphocyte ratios (1:2, 2:1) supported Con.A induced lympho-proliferation while suppression of the response occurred when the ratio of macrophages was increased to 4:1 (Schuyler and Todd, 1981). These discrepancies may be due to the fact that macrophage depletion of the lymphocyte population was not carried out in the former study but that sephadex G10 columns were used in the second study for macrophage depletion.

Canine alveolar macrophages may suppress lymphoproliferation (Kaltreider, 1982) or function as accessory cells in cultures of thymocytes stimulated with Con.A (Wulff et al., 1983). Antigen presentation was shown to be genetically restricted as the Ia positive cells functioned as accessory cells and anti Ia inhibited this accessory cell function.

5.5.2 Human studies

At low concentrations (1%) human alveolar macrophages suppressed the proliferation of peripheral blood lymphocytes, both to mitogens and antigens (McCombs et al., 1982). This suppression was greater with T cells than B cells and appeared to be due to prostaglandin synthesis as indomethacin reversed the inhibition. Another study showed that human alveolar macrophages enhanced mitogen stimulated but not antigen stimulated lymphocyte proliferation and that they are less effective than autologous monocytes when the optimal concentration of PHA was used (Ettensohn and Roberts, 1983). Human blood monocytes were shown to promote T lymphocyte responses when present at low concentrations and suppress proliferation when they comprised more than 30% of the total cell concentration (Twomey et al., 1983). In the same study alveolar macrophages showed a similar pattern but were less efficient as accessory cells and caused suppression at a lower cell concentration than blood monocytes. Generally alveolar macrophages appear to be inferior to peripheral blood monocytes in supporting antigen-induced proliferation. However it was shown that in some individuals the alveolar macrophages had the same activity as monocytes, other individuals possessed alveolar macrophages that suppressed lymphocyte responses, while others had cells that exhibited 15% of the activity of the monocytes (Toews et al., 1984). Therefore some alveolar macrophages present antigen, some cause suppression and some remain inert. The factors that determine which function is expressed in vivo may determine the pulmonary response to inhaled antigen. A high proportion of human alveolar macrophages (80%) express HLA-DR

antigens, therefore they would be expected to be capable of antigen presentation (Mason et al., 1982).

The majority of studies support the concept that the alveolar macrophage is not simply a scavenger cell but is capable of participating in cell-mediated immunologic reactions in the lung. As the first cell in the lung to contact antigen, its role as an accessory cell would be important in the modulation of the host response to antigenic exposure.

5.6. Activation

There is evidence that an effective cell-mediated immune response depends on the recruitment of peripheral blood monocytes into the alveolar spaces (Truitt and Mackaness, 1971) and the activation of the resident alveolar macrophage population (Johnson et al., 1975). The state of macrophage activation is characterised by increased spreading of the cell on glass surfaces, increased numbers of mitochondria and enzymes and an increase in the metabolic activity, phagocytic capacity and intracellular killing by the macrophage (Lasser, 1983). Activation of the alveolar macrophage can be induced directly by appropriate stimuli in vivo, such as bacterial lipopolysaccharide (Sone and Fiddler, 1980), Bacillus Calmette-Guerin (BCG), (Schuyler and Steinberg, 1982) or Freunds adjuvant (Arend and Mannik, 1973), or indirectly by T lymphocytes (Moore and Myrvik, 1977). In vivo macrophage activation results from interaction with lymphokines that are products of stimulated T cells. Intratracheal inoculation of hamsters with Freunds adjuvant (Zwilling and Campolito, 1981) or rabbits with BCG (Schuyler and Steinberg, 1982) was shown to increase the number of alveolar macrophages that possess comple-

ment and IgG receptors and the avidity of the receptors on individual cells. These activated macrophages were also shown to increase in size, exhibit an increased phagocytic capacity and become tumoricidal. During pulmonary infection, alveolar macrophages become activated with a concomitant increase in the clearance of the organism from the lungs.

5.7 Alveolar-lining material

The cell-free portion of broncho-pulmonary lavage fluid has been termed the alveolar-lining material of the lung. This material is important in promoting the bactericidal activity of the alveolar macrophage (LaForce et al., 1973), can produce directional migration of the macrophage and may aid in lung clearance (Schwartz and Christman, 1979). The factor responsible for this activity is a lipid related to surfactant and is not present in the serum (Juers et al., 1976). The lipid material coats the organisms and after phagocytosis has occurred, undergoes peroxidation to produce a bactericidal environment within the phagocytic vesicles (Mason et al., 1972; LaForce et al., 1973; Robertson, 1980). In vitro studies have indicated that alveolar macrophage function is severely impaired in the absence of this material (Skornik et al., 1973; Juers et al., 1976).

6.0 SURFACE RECEPTORS ON ALVEOLAR MACROPHAGES

The macrophage membrane has been shown to express more than thirty different receptors, including receptors for immunoglobulins, complement components, hormones and other particles. This study concentrates on the non-immune "lectin-like" receptor and the Fc receptor for IgG and only these receptors will be reviewed.

6.1 Lectin-like receptors

Lectin-like receptors have the specific ability to bind unopsonised bacteria by means of their cell wall sugars. They are present on mouse peritoneal and alveolar macrophages, human monocytes and neutrophils and a wide range of phagocytes from different species (Glass et al., 1981). Lectin-like receptors are among a group of receptors that recognise carbohydrates, especially sugars,which have been described over the last few years (reviewed by Weir, 1984; Weir et al., 1984; Sharon, 1984).

Lectin-like receptors were first recognised in studies on the interaction between Corynebacterium parvum and mouse peritoneal macrophages. Magnesium and calcium ions are essential for binding of the bacteria to the macrophage, which is inhibited by various sugars (Ogmundsdöttir and Weir, 1976). The ability of a particular sugar to inhibit bacterial binding was further found to be directly related to its presence on the bacterial cell-wall lipopolysaccharide (Freimer et al., 1978). The lectin-like receptor is capable of binding tumour cells and proliferating mouse embryo fibroblasts as their attachment is inhibited by various monosaccharides (Weir et al., 1979). The receptor

is susceptible to proteolytic and glycolytic enzymes, including trypsin, pronase and B galactosidase and also to periodate, although bacterial binding is increased by neuraminidase (Ogmundsdöttir et al., 1978). The action of trypsin was found to be reversible with bacterial binding returning to normal levels after a few hours. The sensitivity of the receptor to these enzymes, indicates that it is a glycoprotein. Conversely bacteria may bind to macrophages via a lectin present on the bacterial cell wall. C.parvum contains a mannose specific lectin that binds to macrophages that possess mannose on their cell membrane (Bagg et al., 1981). However sugars do not inhibit binding when incubated with bacteria (Ogmundsdöttir, PhD thesis, 1979).

6.1.1 Function

The binding of bacteria to macrophages is an essential process for the initiation of phagocytosis and antigen presentation (Unanue, 1978). The sugar recognising lectin-like receptor is involved with other binding mechanisms (Capo et al., 1981) in the initial binding of unopsonised bacteria to phagocytes prior to the involvement of Fc or C3 receptors and antibody production. Therefore these receptors are possibly important in the recognition and destruction of unopsonised bacteria before an immune response is initiated. An in vivo study has shown that peritoneal macrophages from diabetic mice bind fewer bacteria than macrophages from non-diabetic mice (Weir et al., 1981). These results indicated that sugar molecules were blocking lectin-like receptors on the macrophages of the mice, that were subsequently more susceptible to bacterial infection. The lectin-like receptor may also be important in antigen presentation as it has been shown to be

associated or identical with IA antigens (Stewart et al., 1983). Various monoclonal antibodies to IA antigens were shown to inhibit the binding of bacteria to macrophages. Further evidence of a role for this receptor in antigen presentation comes from studies on mice with alloxan-induced diabetes. Peritoneal macrophages of these mice were less efficient at binding Staph.albus and at antigen presentation than macrophages from untreated mice. However Fc receptor expression was not altered in the diabetic mice, arguing against a general malfunction of the macrophages from these animals (E J Glass, personal communication).

Lectin-like receptors may also be involved in macrophage recognition of altered self, including transformed cells (Weir et al., 1979) and effete red blood cells (Kolb and Kolb Bachofen, 1978).

6.1.2 Regulation

In vitro exposure of mouse peritoneal macrophages to chemotactic agents, F Met Leu Phe and casein, and in vivo exposure to oyster glycogen and C.parvum inhibited bacterial binding activity (Glass et al., 1982, 1983). Similar findings were observed for the receptor on mouse macrophages specific for mannose, fucose and N-acetylglucosamine (Ezekowitz and Gordon, 1982; Imber et al., 1982). BCG and C. parvum activated peritoneal macrophages showed reduced binding and uptake of radio-labelled mannose.

Pseudomonas alginate has been shown to inhibit the binding of P.aeruginosa and Staph.albus to mouse peritoneal and pulmonary macrophages (Oliver and Weir, 1983). Further studies on the lectin-like receptor

will be described in this thesis.

6.2 Fc receptors

Fc receptors bind the Fc portion of immunoglobulin and are usually identified by rosette formation with antibody labelled red blood cells.

Fc receptors have been identified on a number of different cells, including B lymphocytes (Dickler and Kunkler, 1972), monocytes (Abramson et al., 1970a,b) and neutrophils (Ishizaka et al., 1970). Human promonocytes and bone marrow monocytes, progenitors of the tissue macrophage also possess Fc receptors (van Furth et al., 1979).

Alveolar macrophages possess receptors for the Fc portion of IgG (Reynolds et al., 1975a; Boltz-Nitulescu et al., 1981) and IgE (Boltz-Nitulescu and Spiegelberg, 1982), but not IgM. Human alveolar macrophages are capable of phagocytosis of bacteria opsonised with IgA, indicating the presence of an Fc receptor for this immunoglobulin (Reynolds et al., 1975a). A study on mice has shown that 14% of their alveolar macrophages possess an Fc receptor for IgA, that increases to 30% on activation (Gauldie et al., 1983). This is not surprising as IgA is the principal immunoglobulin in the normal lung.

6.2.1 Avidity

The avidity of Fc receptors increases as monocytes differentiate into macrophages in culture, although the response initially drops between one and three days of culture (Newman et al., 1980). The avidity of Fc receptors increases as macrophages become activated and this change may be used to differentiate macrophages into subpopulations

(Moore and McBride, 1980). The cells with the highest avidity Fc receptors are identified as the most highly activated cells in the population. The mean avidity may also differ between populations of macrophages at different sites within an animal. For example guinea-pig peritoneal macrophages have a Fc receptor avidity three times that of their alveolar macrophages (Rhodes, 1975). Similarly rat and murine alveolar macrophages have low avidity Fc receptors when compared to peritoneal macrophages (personal observation), although subpopulations within the lung may possess very high Fc receptor avidity (Zwilling et al., 1982).

The degree of adherence of soluble complexes to Fc receptor bearing cells was shown to be directly correlated with the affinity of the receptor site and not the absolute number of receptors (Arend and Mannik, 1973). This suggests that the receptors may vary in their availability or accessibility on the cell membrane and that this may be influenced by their orientation with respect to adjacent surface proteins. An intact covalently bound Fc fragment is essential for binding and requires the presence of the CH2 domain (Diamond et al., 1979; Dorrington and Klein, 1982).

6.2.2 Turnover

Receptor mediated phagocytosis leads to a selective and largely irreversible removal of Fc receptors from the macrophage plasma membrane. Interiorised Fc receptors are rapidly and selectively degraded. In resting cells, receptor turnover has a half life of ten hours. However after phagocytosis more than 50% of the receptors are

degraded and the half life is reduced to two hours (Mellman et al., 1983).

6.2.3 Function

The main function of the Fc receptor is to augment the phagocytic uptake of viable bacteria when they are specifically coated with antibody (Reynolds et al., 1975a). In monocytes and macrophages, the Fc receptor facilitates the phagocytosis of foreign (Reynolds et al., 1974) and autologous material (Kay, 1975). The receptor is also able to mediate antibody dependent cell mediated cytotoxicity (ADCC)(Haskill and Fett, 1976; Shen and Fanger, 1981), stimulate superoxide anion production (Goldstein et al., 1975) and the release of many other factors, including prostaglandins (Bonney et al., 1980).

6.2.4 Specificity and type

The most detailed studies have been carried out on murine Fc receptors and three distinct IgG Fc receptors have been described. FcRI binds monomeric and aggregated IgG2a with high affinity and is sensitive to trypsin, cytochalasin B and the cold (4°C) while FcRII binds aggregated IgG2b and antigen bound IgG1 and is resistant to trypsin, cytochalosin B and the cold (Diamond et al., 1978). FcRIII binds IgG3 and is similar to FcRII in that it is resistant to trypsin, cytochalasin B and the cold (Diamond and Burshstein, 1981). The receptor for IgG3 appears to be a separate receptor as IgG1, IgG2a and IgG2b aggregates do not compete with IgG3 aggregates for binding. All three receptors mediate phagocytosis and ADCC of opsonised sheep erythrocytes (Unkeless et al., 1979; Diamond et al., 1978; Ralph

et al., 1983). Similarly rat macrophages have at least two distinct IgG Fc receptors. One receptor is specific for IgG2a and is resistant to trypsin, while the other receptor reacts with IgG1, IgG2b and heterologous IgG and is sensitive to trypsin (Boltz-Nitulescu et al., 1981). These receptors mediate phagocytosis and lysis.

Human IgG3 consistantly bound to human alveolar macrophages more than the other subclasses, IgG1, IgG2 and IgG4, indicating the presence of a specific Fc receptor for IgG3 on these cells (Naegel et al., 1984).

6.2.5 Number/cell

There are between 10^5 and 10^6 Fc receptors per mononuclear phagocyte although the number varies between species (Unkeless and Eisen, 1975; Alexander et al., 1978) and activated macrophages may have more receptors as a result of increased cell size (Newman et al., 1980). Heavily stimulated rabbit alveolar macrophages were found to possess 2.16×10^6 receptor sites for IgG per cell, almost double the number (1.21×10^6) found in unstimulated cells (Arend and Mannik, 1973; Newman et al., 1980). A comparative study showed that guinea-pig peritoneal macrophages had fifty times the number of Fc receptors (10^6/cell) than neutrophils (2×10^4/cell) (Coupland and Leslie, 1983).

6.2.6 Isolation and characterisation

Two polypeptide chains from a mouse macrophage cell line have been isolated with molecular weights of approximately 60,000 and 47,000 daltons that recognised IgG2a and IgG2b respectively (Mellman and

Unkeless, 1980). Similarly two receptors have been isolated from thioglycollate stimulated macrophages (Lane et al., 1980). One protein had FcRI activity and a molecular weight of 67,000 daltons while the second had FcR-II activity, and a molecular weight of 54,000 daltons. An FcR-like protein was isolated from rabbit alveolar macrophages with the use of sepharose affinity columns (Kulczycki et al., 1980). However the molecular weight of the compound varied with the gel, being 50,000 - 70,000 daltons in 5.6% acrylamide gel and 35,000-55,000 daltons in 9% acrylamide gel.

An Fc receptor protein isolated from the human cell line U937 formed two bands on polyacrylamide gel of molecular weights 72,000 and 40,000 (Anderson, 1982). The former protein bound IgG1 but not IgG2. The predominant Fc receptor isolated from plastic-adherent human blood monocytes was shown to have a molecular weight of 60,000 - 68,000 daltons by SDS-polyacrylamide gel electrophoresis (Cohen et al., 1983).

6.2.7 Regulation

The number of IgG receptors on rabbit alveolar macrophages was shown to increase after intratracheal injection of complete Freuds adjuvant (Arend and Mannik, 1973) or BCG (Montarraso and Myrvik, 1978) into the lung. Similarly IgG2b receptor expression was found to be slightly increased in thioglycollate stimulated mouse macrophages and markedly increased in C.parvum activated macrophages (Glass et al., 1983). Incubation of peritoneal macrophages with lung lining material causes the cells to lose their Fc receptors (Zeligs et al., 1984). Thus the environment can influence the expression of macrophage Fc

receptors.

Modulation of Fc receptors on rat alveolar macrophages and human monocytes by antibiotics is investigated in this thesis.

6.3 Complement receptors

Receptors for C3b and C3d, the third components of complement are present on bone marrow monocytes (van Furth et al., 1979), monocytes and most normal alveolar macrophages (Daughaday and Douglas, 1976; Warr and Martin, 1977). However alveolar macrophages from Sprague-Dawley, Long Evans and Fisher rats were shown to lack receptors for C1, C2 or C3 although they were present on Lewis-Wistar strains (Coonrad and Rehm, 1982). Peritoneal macrophages from these strains of rats were shown to possess receptors for C1 and C3 and were able to bind bacteria opsonised with complement. The reason for the lack of receptors on the alveolar macrophage is unknown, however peritoneal macrophages treated with rat lung lavage fluid exhibited a loss in complement receptor function (Coonrad and Yoneda, 1983). This effect was reversible and it was postulated that lipids in the alveolar lining material affected alveolar macrophage membrane and receptor functions. Swiss mice have also been shown to lack C1 and C3 receptors on their alveolar macrophages (Hearst et al., 1980).

Complement levels are low in the lung (Reynolds and Thompson, 1973) so that alveolar macrophage C3 receptors may not be functionally important in vivo. The early phase of killing of Pneumococci in human alveoli does not require complement (Rehm and Coonrad, 1982).

Phagocytosis of complement coated sheep erythrocytes was low in human monocytes, although peritoneal macrophages did ingest a high proportion of the erythrocytes, indicating that binding via C3 receptors may only cause phagocytosis by activated macrophages (Newman et al., 1980). Human alveolar macrophages were able to phagocytose bacteria opsonised with activated C3 in the absence of IgG (Richards et al., 1984). C3b and Fc receptors may act synergistically, for although adhesion of particles to the C3b receptor does not necessarily result in phagocytosis (Fearon, 1984) adhesion may cause an increase in the uptake of IgG coated particles (Ryter and DeChastellier, 1983).

7.0 PHAGOCYTOSIS

7.1 Definition

Phagocytosis is an endocytic mechanism and involves the uptake of large particles (71 micron) such as micro-organisms, latex beads and oil droplets by an active receptor-mediated process. Phagocytosis is clearly distinguished from pinocytosis, an endocytic mechanism that involves the uptake of small particles or soluble material such as ferritin, macromolecules and low molecular weight solutes. Pinocytosis is a mainly passive process that occurs by membrane invagination, the concentration gradient determining the rate of particle transfer (Chapman-Andersen, 1977).

Phagocytosis can be divided into two distinct events, attachment or adhesion of a particle to the cell surface, which is followed by ingestion. While the first process occurs in the cold and does not require metabolic energy, ingestion is temperature dependent and requires active cellular metabolism to occur (Rabinovitch, 1967; Ito *et al.*, 1981).

7.1.1 Attachment

The initial event of the phagocytic process is the contact and adhesion of particles to the cell surface. Adherence may occur as a result of nonspecific interactions between the particle and the cell (Bongrand *et al.*, 1982) or specific receptor-mediated interactions.

Adhesion of positively charged particles is stronger than that of negatively charged particles on account of repulsive forces (Weiss, 1969) and is enhanced by the removal of negative ions from a particle, such as an erythrocyte (Capo *et al.*, 1979). However electrostatic

forces do not play a main role in particle adhesion as bacteria and macrophages are often both negatively charged (Beveridge, 1980). Adhesion of latex, oil droplets and glutaraldehyde-fixed cells to macrophages takes place by means of unspecific binding sites (Stossel, 1975; Vogel et al., 1980; Benoliel et al., 1980). In many cases this unspecific binding seems to be a result of the hydrophobicity of the particles or molecules (Wilkinson, 1976; van Oss, 1978). High interfacial tension between the particle and surrounding medium, but low interfacial tension against the phagocytic cell favours binding (van Oss, 1978). Cationic substances enhance adhesion as they favour the formation of large contact areas between the particle and phagocytes (Capo et al., 1981).

Specific receptors that mediate attachment include "lectin-like" receptors, Fc and complement receptors and have been discussed.

7.1.2 Ingestion

Ingestion involves the formation of membrane extensions that surround and finally fully enclose the particle in a phagocytic vacuole. This process is rapid at optimum conditions and may occur within 10-30 seconds after the initial contact of the particle (Bowers, 1980; MacRae et al., 1980). It is temperature dependent (Silverstein et al., 1977), requires metabolic energy and is promoted by calcium ions (Stossel, 1975; Hartwig et al., 1980).

7.2 Mechanism

The "zipper" mechanism of phagocytosis proposed by Griffin et al.,

(1975b) involves the ligand-mediated circumferential spreading of the plasma membrane around a particle. Phagocytosis will only occur if macrophage membrane receptors, that were not involved in the initial attachment process, bind to ligands over the whole surface of the particle (Griffin et al., 1975b, 1976). Following attachment of a particle to the Fc receptor, thin lamellipodia form and remain tightly apposed to the particle surface throughout the engulfment process (Stossel and Hartwig, 1976; Silverstein et al., 1977). Pseudopods that form around the particle form a large depression, termed the phagocytic cup. This gives rise to a phagosome upon closure of the membrane and fuses with lysosomes to form secondary lysosomes in which internalised material is digested by acid hydrolases (review by Silverstein et al., 1977). However the "zipper" mechanism has not been shown for all receptors (Kaplan, 1977; MacRae et al., 1980; Ryter and Hellio, 1980). Ingestion will occur when contact areas are randomly located along pseudopods or filapods (MacRae et al.,1980; McNeil et al., 1981) and C3 receptors establish discontinuous contacts (Kaplan, 1977).

7.2.1 Contractile Mechanism

Cytoplasmic contractile elements provide the locomotive force for advancing pseudopods (filapodia or lamellipodia). The submembrane region adjacent to the particle being ingested contains a thick network of filaments, from which cytoplasmic organelles are excluded (Griffin et al., 1976). This network contains actin, myosin and an actin-binding protein (Stendahl et al., 1980; Valerius

et al., 1981). Purified actin and actin binding protein from alveolar macrophages form gel lattices at low calcium concentrations, while myosin, in the presence of magnesium ions and ATP causes isometric contraction of the actin filaments (Stossel and Hartwig, 1976). A calcium dependent regulator protein, gelsolin, has been identified and shown to control the formation of the actin - actin binding protein lattice (Yin and Stossel, 1980). Contraction of the actin filaments that are attached to the membrane leads to the membrane being pinched upwards and outwards, to form a pseudopod (Hartwig et al., 1977). An increase in the calcium concentration in the presence of gelsolin results in the dissolution of actin - actin binding protein lattice. Thus as the region of rigid membrane moves round the particle, it is followed by a secondary wave of relaxation. A gradient is maintained, with rigidity being maximal in the tip of the advancing pseudopodium. Throughout the process fresh insertion of the membrane takes place, to keep pace with the process of internalisation (Ryter and De Chastellier, 1977).

7.3. Triggering mechanism

The mechanism that triggers the contractile protein mobilisation is not clearly understood. The stimulus to phagocytose a bound particle does not result in the ingestion of other particles that are attached to the membrane surface (Griffin and Silverstein, 1974; Griffin et al., 1975a). The response of the membrane is confined to that segment of membrane immediately adjacent to the particle initiating the stimulus, so that the signal for phagocytosis is restricted to that particle. It has been proposed (Griffin et al.,

1976) that in the case of IgG coated particles, the ligand-receptor interaction generates the release of actin-binding protein from the plasma membrane. The protein is then believed to initiate polymerization and aggregation of contractile proteins which leads to the extension of phagocytic pseudopods. Pseudopod extension causes further receptor-ligand interaction, which consequently generates further contractile protein association. However this hypothesis cannot account for the many cases in which contacts between a particle and the phagocytic membrane are loose and discontinuous (MacRae et al., 1980). It is therefore likely that the signal triggering contractile protein mobilisation can propagate in the absence of a progressive and permanent contact between particle and phagocyte. The factors triggering the dissociation of the filament network, once internalisation has occurred, also remain unknown (Ryter and De Chastellier, 1983). It appears that phagosome closure is not a prerequisite for local microfilament network dissociation (De Chastellier and Ryter, 1982).

After heavy phagocytic activity, phagocytes enter a refractory period, during which particle uptake is markedly suppressed (Werb and Cohn, 1972). Cells round up and are incapable of further phagocytosis for several hours. This refractory period appears to be due to the time required to replace the interiorised plasma membrane (Low, 1975).

8.0 ANTIBIOTICS IN CYSTIC FIBROSIS

The improvement in the prognosis of CF patients can to a large extent be ascribed to the introduction and development of more potent antibiotics since the 1940's. For example, penicillin and streptomycin in the 1940's, tetracycline, chloramphenicol and erythromycin in the 1950's, ampicillin, carbenicillin and gentamicin in the 1960's; tobramycin and a range of second and third generation cephalosporine and ureidopenicillins in the 1970's and 1980's (Høiby et al., 1982).

The principal beneficiaries of antimicrobial therapy are normally healthy people who have an acute bacterial infection, whereas patients with CF who have chronic respiratory infection are rarely cured by antimicrobial therapy. In the latter situation, antibiotics may control the infection, but therapy is often frequent and prolonged. Therefore any effect that an antibiotic may have on the host, other than an antimicrobial one, may be clinically important.

Three antibiotics used extensively in the therapy of CF patients, namley azlocillin, ticarcillin and tobramycin have been studied for their effects on the alveolar macrophage. These antibiotics all improve the clinical status of the patients but mucoid P.aeruginosa are rarely eradicated (Marks et al., 1976). Their structure and antimicrobial activity are discussed below.

8.1 Azlocillin

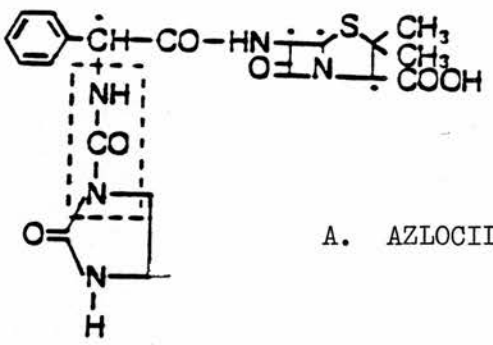
Azlocillin is a semi-synthetic B-lactam derived from ampicillin. It possesses an acyl ureido derivative alteration at the amino group

of the ampicillin molecule (Diagram 2).

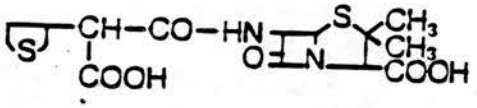
8.1.1 Antibacterial activity

As a B-lactam, azlocillin inhibits the growth of bacteria by blocking the action of transpeptidases essential for the crosslinking of the bacterial cell wall (Tipper and Strominger, 1965) so that the bacteria must be multiplying for the antibiotic to exert its bactericidal activity. Recently several additional enzymes, termed penicillin-binding proteins (PBP), essential for cell wall synthesis have been found to be inhibited by B-lactams. P.aeruginosa possess eight PBPs (Prince and Neu , 1981). The binding affinity of an antibiotic for the receptor protein will in part determine the inhibitory concentration of a particular B-lactam. The possession of an acyl ureido group gives azlocillin an increased affinity for critical penicillin-binding proteins, particularly the enzyme, septal murein synthetase. The enzyme is responsible for septal formation during bacterial growth and cell division. Inhibition of the enzyme results in the formation of nonviable and readily lysed filamentous bacterial cells.

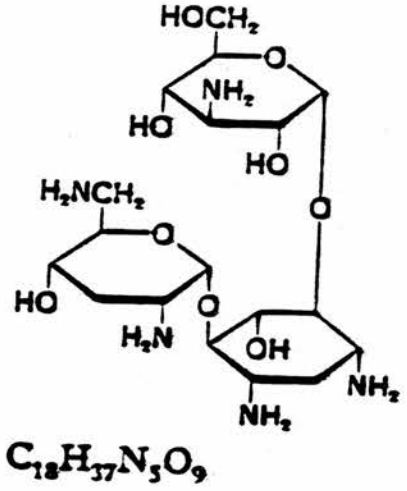
The high activity of azlocillin is due to the antibiotics' ability to pass through the outer bacterial cell wall and bind to the target proteins with such high affinity that the penicillinase enzymes cannot degrade it rapidly enough (Prince and Neu, 1983). Azlocillin is more active in vitro against P.aeruginosa than ticarcillin (Coppens and Klastersky, 1979) or carbenicillin (Hodson and Batten, 1981) and has an MIC range, for the bacteria,of 4-32 mg/l (Sanders, 1983).



A. AZLOCILLIN



B. TICARCILLIN



$C_{18}H_{37}N_5O_9$

C. TOBRAMYCIN

Diagram I Structure of the three antibiotics used in this study.

Azlocillin is not normally used as a single agent for the treatment of P.aeruginosa infection because of the risk of resistance (Michalsen and Bergan, 1980; Nørrby, 1981).

8.2 Ticarcillin

Ticarcillin is an α- carboxy-3-thienyl methyl penicillin and lacks a ureido group (Diagram 2).

8.2.1 Antibacterial activity

The antibacterial mechanism of action of ticarcillin is similar to that already described for azlocillin, although it is less active in vitro than azlocillin as ticarcillin lacks a ureido group. However its activity against P.aeruginosa exceeds that of carbenicillin by two to four fold (Comber et al., 1977).

Using electron microscopy it was found that azlocillin and ticarcillin at similar concentrations initially caused the formation of filaments by P.aeruginosa (Elliott and Greenwood, 1983). On further incubation deposits of dense intracellular material were observed with subsequent lysis of the filaments. Azlocillin caused breakages at a restricted number of sites on the cell wall, while ticarcillin caused breakage at many points. Therefore, ticarcillin may act more rapidly and be more efficient at causing bacterial lysis than azlocillin. The MIC of ticarcillin for P.aeruginosa is in the range 11.2-62.4 (Coppens and Klastersky, 1979).

8.3 Tobramycin

Tobramycin is an aminoglycoside and contains amino sugars

glycosidically linked to aminocyclitols (Diagram I).

8.3.1 Antibacterial activity

Tobramycin is rapidly bactericidal and acts on the ribosomal subunit to block bacterial protein synthesis. It is highly active against P.aeruginosa being four times more active than gentamicin, that has generally superseded in the treatment of P.aeruginosa infections (Norrby, 1981). Tobramycin remains active against strains with acquired resistance to gentamicin (Hyams et al., 1973; Hodson and Batten, 1981) and the susceptibility of this antibiotic has remained unchanged compared to gentamicin (Blessing et al., 1981). Although tobramycin can eliminate non-mucoid P.aeruginosa, mucoid forms are only reduced in number (Hoogkamp-Korstanje and van der Laag, 1983).

Tobramycin is fairly resistant to the inactivating bacterial enzymes, such as phosphorylases, adenylases and acetylases that occur in P.aeruginosa strains and this may account for its high anti-pseudomonal activity. The MIC against P.aeruginosa is in the range 0.25-4 mg/l (Rolston et al., 1984).

Tobramycin acts synergistically with the B-lactam antibiotics and helps to prevent the development of P.aeruginosa resistance to the B-lactams, such as azlocillin (Hoogkamp-Korstanje et al., 1981). The phenomenon of synergy between aminoglycosides and B-lactam antibiotics against P.aeruginosa can be explained by the activity of the B-lactam on the cell wall, facilitating the penetration of the aminoglycoside. Synergism requires that both the antibiotics in combination are present at optimal concentrations at the site

of an infection (Zak, 1980).

8.4 Antibiotic therapy in Cystic Fibrosis

Antibiotic treatment of mucoid P.aeruginosa pulmonary infection in CF patients is usually ineffective as the organisms are rarely eradicated (Kulczycki et al., 1978). In fact it has been claimed that the continuous use of antistaphylococcal antibiotics in CF has contributed to the increasing isolation of P.aeruginosa (Kulczycki et al., 1978).

Although the levels of anti-staphylococcal and anti-haemophilus antibiotics achieved in the CF lung are able to inhibit bacterial growth, it is thought that adequate levels of anti-pseudomonal drugs are rarely achieved (Raeburn, 1975). Four hours after an intravenous infusion, azlocillin was only detected in 34 out of 111 sputum samples, where the concentration range (0.5-4 mg/l) was below the MIC range for P.aeruginosa. However the antibiotic concentration in serum was markedly higher (32-142 mg/ml) (Levy et al., 1982). Another study showed that the sputum antibiotic concentrations were low (1.5-28 mg/ml) after a large intravenous injection of azlocillin (Malmborg et al., 1981). Similar studies with tobramycin have shown the concentration in sputum to be below the MIC (0.1 - 0.4 mg/l) after intravenous injection of large doses (Malmborg et al., 1981). Tobramycin was shown to penetrate slowly into the sputum but to give fairly constant levels of up to 13% of peak serum level (0.57-0.68 mg/l) (Hoogkamp-Korstanje and van der Laag, 1983). Treatment of patients was most successful in whom the highest peak sputum concentration of tobramycin was obtained, as opposed to the absolute dose given

(McCrae et al., 1976). High doses of tobramycin were shown to eradicate P.aeruginosa from four out of seventeen patients. However the concentration of B.lactam antibiotics in sputum did not predict the outcome of the treatment, as high levels did not correlate with elimination of the bacteria (Malmborg et al., 1981). Inhalation of tobramycin resulted in a higher percentage of eradication than intravenous treatment (Stephens et al., 1983). However, in this study the observed suppression of P.aeruginosa in the sputum did not correlate with the clinical response to treatment. This response did not differ between the two groups and eradication of the bacteria was only temporary with inhalation studies. These results illustrate the complexicity of the problems involved in the treatment of P.aeruginosa infection in CF.

Although many factors are involved in the persistent P.aeruginosa infection observed in CF, the low levels of antibiotics attained in the lungs of these patients may in part account for the ineffective eradication of this organism. As previously discussed, CF patients with chronic infection may be immunocompromised or hyper-immune, so that antibiotic therapy may be less efficient and quite different for CF than normal individuals. Information on the effective antibiotic concentration at the site of infection, distribution and half-life in pulmonary secretions is limited, making treatment difficult. Concentrations of antibiotic in the sputum may not reflect the concentration at the site of infection. Similarly sputum cultures and bacterial numbers do not always correlate with that obtained from lung culture (Doershuk et al., 1976).

Purulent material such as pus may inactivate antibiotics or they may bind pus or P.aeruginosa alginate and become ineffective (Bryant and Hammond, 1974). Diffusion of aminoglycosides, though not B.lactams has been shown to be retarded in vitro by P.aeruginosa alginate and sodium alginate (Slack and Nichols, 1981). It was postulated that this result was due to the negatively charged alginate acting as a cation exchanger to inhibit the diffusion of positively charged antibiotics, such as the aminoglycosides.

Antibiotics are eliminated more rapidly in CF patients than normal individuals (Marks, 1981; Kelly et al., 1982) so for serum levels to be maintained, higher doses than those prescribed may be required for the CF patient (Smith, 1980). Pharmacokinetic studies have demonstrated altered deposition, increased excretion and low peak serum levels of antibiotics in CF patients treated with conventional doses of semisynthetic penicillins (Prince and Neu, 1980) and aminoglycosides (Finkelstein and Hall, 1979).

Improvement in pulmonary function does not always correlate with the bacteriologic response of the patient. Factors, other than intravenous antibiotic therapy, may contribute to the beneficial response observed during hospitalisation (McLaughlin et al., 1983).

Antibiotics may be prescribed intermittently to treat specific exacerbations identified clinically and by sputum bacteriology or prophylactically and continuously (Møller and Høiby, 1981). Both regimens have disadvantages as there is a fall in the efficacy of an antibiotic when antipseudomonal treatment is repeated. Prophylactic treatment may result in a reduced capacity to resist secondary bacterial challenge and the emergence of resistant strains (North et al., 1981).

9.0 THE EFFECT OF ANTIBIOTICS ON THE IMMUNE RESPONSE

Since chemotherapy became established, the direct effect of antimicrobial substances on bacteria have been extensively studied, with little attention being given to the effects on the host's immune defense system. Conventional antimicrobial sensitivity testing only considers the interaction between the drug and the microorganism, ignoring the possible effects of humoral and cellular factors on this relationship. Yet for antibiotic treatment to be effective in the long term, an adequate host immune response is essential (Peterson, 1982). Antibiotics alone do not eliminate bacteria, they either kill or retard their growth, leaving the host to clear the organisms and toxic products from the body.

Subinhibitory concentrations of antibiotics have been shown to induce morphological and biochemical changes in bacteria (Lorian, 1975) and render them more susceptible to phagocytosis and killing (Gemmell et al., 1981; Milatovic, 1982). The effect of these low antibiotic concentrations may be important as drug levels achieved in the tissues may not attain the MIC of the target organism, as previously discussed.

Adverse effects of an antibiotic on the immune system may be difficult to identify as the effect may be transient, easily reversed or attributable to the primary infection (Raeburn, 1982). During an acute bacterial infection, any disadvantageous effect of an antibiotic would be likely to be outweighed by the benefits of the drug in eliminating the organisms. Therefore the relevance of many

observations is probably doubtful as antimicrobial therapy is usually short-lived. However treatment of chronic bacterial infections such as those that occur in CF require continuous or intermittant therapy. In this case any effect that an antibiotic may have on the host immune response may be important and influence the progress of an infection. Immune mechanisms play an integral part in the pathogenesis of CF and are important for recovery. Any immuno-modulating effect of an antibiotic may have therpeutic significance and an awareness of potential effects on the immune system could be important. The many effects that antibiotics have on the immune system have been reported and reviewed (Hauser and Remington, 1982a,b; Mandell, 1982; Milatovic, 1983; Oleske, 1984) and will be discussed below. Observations at concentrations that exceed those achieved therapeutically are unlikely to have clinical significance (Finch, 1980) and will not be considered in this review.

9.1 Neutrophils

Initially neutrophils are the first cells to migrate and concentrate at the site of bacterial infection, therefore any interaction with antibiotics may be very important (Raeburn et al., 1980). Many studies have been performed on the neutrophil as opposed to the macrophage, possibly because neutrophils are easily obtained from human peripheral blood (review by Mandell, 1982).

8.1.1 Tetracyclines and others

Tetracyclines have been shown to inhibit neutrophil chemotaxis (Forsgren and Schmelling, 1977; Belsheim and Gnarpe, 1981),

phagocytosis (Forsgren et al., 1974; Gnarpe and Belsheim, 1981), intracellular killing (Welch et al., 1981) and adherence to glass (Gnarpe and Belsheim, 1981). Rifampicin and trimethoprim were also shown to depress neutrophil chemotaxis (Forsgren et al., 1980; Welch et al., 1981), chemiluminescence (Siegel and Remington, 1982) and intracellular killing (Easmon, 1979; Welch et al., 1981).

9.1.2 B.lactams

Generally the penicillin and cephalosporin antibiotics have not been shown to have any effect on neutrophil function (Forsgren and Schmelling, 1977; Majesky et al., 1978; Gnarpe and Belsheim, 1981; Hawkey et al., 1983). However, there are a few exceptions. At concentrations below the MIC, carbenicillin and sulbenicillin were shown to increase the phagocytosis and intracellular killing of P.aeruginosa by rabbit neutrophils (Nishida et al., 1976). In vivo ampicillin depressed while cefaclor enhanced the myeloperoxidase activity and phagocytosis of Staph.aureus by human neutrophils (Kowolik et al., 1982; Grant et al., 1983).

Ceftazidine was shown to increase neutrophil adherence to nylon wool in vitro (Gnarpe et al., 1984) and in vivo to increase the chemiluminescence of human neutrophils stimulated with opsonised zymosan, but not opsonised Staph.aureus (Cullen et al., 1983). A new semisynthetic cephalosporin, AC 1370, has been found to augment the chemotaxis, NBT reduction, phagocytosis and intracellular killing in murine and human neutrophils (Ohnishi et al., 1983).

9.1.3 Aminoglycosides

Results from studies of this group of antibiotics on the immune response have been contradictory and confusing. Using Boyden chambers, aminoglycosides including gentamicin were shown to inhibit the migratory activity of neutrophils (Goodhart, 1977; Belsheim and Gnarpe, 1981), while with studies using an agarose plate method, these antibiotics had no effect on chemotaxis (Foragren and Schmelling, 1977; Forsgren et al., 1980). Similarly other studies have shown that aminoglycosides have no effect on phagocytosis (Welch et al., 1981; LeMoli et al., 1983) or intracellular killing by rabbit (Nishida et al., 1976) or human neutrophils (Welch et al., 1981). Gentamicin however inhibited the intracellular killing of bacteria when added to the neutrophils after ingestion had occurred (Easmon, 1979) but had no effect when added prior to ingestion (Nishida et al., 1976). Seckleki et al., (1978) studied neutrophil migration and adherence in the presence of varying concentrations of amikacin, kanamycin, gentamicin, tobramycin and netilmicin, in vitro. Bacterial chemotactic factor-induced chemotaxis was inhibited by each of the aminoglycosides and was dependent on the continual presence of the antibiotic. Adherence of neutrophils to mylon fibres was moderately increased, while phagocytosis and NBT reduction were unaffected by the antibiotics. Neutrophils incubated with various aminoglycosides including tobramycin, prior to the addition of candida, exhibited decreased candidacidal activity (Ferrari et al., 1980).

In vivo gentamicin and amikacin given to normal healthy adults resulted in a transient decrease in chemotaxis but an increase in

random migration of their neutrophils (Khan *et al.*, 1979). The observed inhibition occurred one hour after the injection but not at 24 hours and did not correlate with the antibiotic concentration in the serum.

9.2 Macrophages

In contrast to the neutrophil, the effect of antibiotics on the macrophage, despite its importance in the immune response has received only limited attention. *In vivo* rifampicin has been shown to inhibit the phagocytic activity of mouse peritoneal macrophages when given intraperitoneally (Bassi *et al.*, 1973). In this case the main target of the antibiotic was shown to be the phagocytosing cell. Ampicillin and tetrocycline were shown to enhance the phagocytosis of *Listeria monocytogenes* by human monocytes (Adam, 1982). Similarly, penicillin, gentamicin, tobramycin and tetracycline were shown to enhance the phagocytosis of *L.monocytogenes* by cultured human monocytes, although ampicillin had an inhibitory effect in this study (Cifarelli *et al.*, 1982). Cephalothin and gentamicin were reported to have no effect on phagocytosis but to increase the intracellular killing of bacteria by guinea-pig alveolar macrophages (Brunner and Undeutsch, 1982). Therefore the effect observed with some antibiotics, for example, gentamicin, may depend on the type of mononuclear phagocyte studied.

When human peripheral blood monocytes were incubated for one hour with ceftazidine, a depressed chemiluminescent response was observed (Cullen *et al.*, 1983). AC,1370 as well as augmenting neutrophil function, was shown to increase the phagocytosis of bacteria by mouse peritoneal macrophages *in vitro* and *in vivo*

(Ohnishi et al., 1983). This antibiotic appears to potentiate phagocytic function as well as having a bactericidal effect.

The effects of azlocillin, ticarcillin and tobramycin on rat alveolar macrophages and human monocytes are investigated in this thesis.

9.3 Lymphocytes

The effect of antibiotics on the humoral and cell-mediated responses has been reviewed (Hauser and Remington, 1982a) and will be briefly discussed below.

An early study showed that tetracycline, erythromycin and penicillin had no effect on human lymphocyte proliferation in response to PHA (Dam et al., 1975). Munster et al., (1977) measured DNA synthesis of PHA stimulated lymphocytes and found that tetracyclines suppressed DNA synthesis in a dose-dependent fashion, while B.lactams had no effect and gentamicin slightly stimulated DNA synthesis. However, another study showed that two B.lactams, moxalactam and cefuroxine depressed the PHA response of lymphocytes when incubated with the cells and PHA (Manzella and Clarke, 1983). Pre-incubation of the lymphocytes with the antibiotics had no effect and the depressive effect was less marked when Con A was the mitogen. Tetracycline and fusidic acid have been shown to inhibit the mitogenic response of T and B lymphocytes, in vitro antibody production and protein synthesis in unstimulated lymphocytes (Banck and Forsgren, 1979; Thong and Ferrante, 1979). These antibiotics have also been shown to reduce the delayed type hypersensitivity reaction to sheep erythrocytes (Thong and Ferrante, 1980). Chloramphenicol was shown to suppress

antigen-induced lymphocyte blastogenesis, but not lymphokine production by stimulated lymphocytes (Damert and Sohnle, 1979).

Generally studies have failed to demonstrate any effect on cell mediated immunity by aminoglycosides or penicillins (Banck and Forsgren, 1979; Hauser and Remington,1982b). However, cephalosporins have been shown to produce a dose-dependent reduction in the human lymphocyte response to PHA, PWM and Con A (Chaperon and Sanders, 1978; Banck and Forsgren, 1979), although ceftazidine has no effect on these functions (Gnarpe et al., 1984). Another study showed that antibiotics including peneicillin and gentamicin reduced in vivo antibody production in rabbits (Lochmann et al., 1979). In vitro gentamicin was shown to have no effect or antibody synthesis (Forsgren et al., 1980).

9.4 Transport into cells

The effect of an antibiotic may be related to its ability to penetrate into the phagocytic cells of the host. Penicillins and cephalosporins are taken up poorly by rabbit and human alveolar macrophages and neutrophils as intracellular levels were shown to be low (Johnson et al., 1980a; Prokesch and Hand, 1982). Similarly aminoglycosides do not attain high intracellular levels although they are taken up to a greater extent than the B.lactams (Prokesch and Hand, 1982; Hand et al., 1984).

Lipid soluble antibiotics such as rifampicin, chloramphericol and the tetracyclines are taken up by alveolar macrophages and concentrated within the cell (Hand et al., 1983). Erythromycin and clindamycin are actively taken up into cells via the nucleoside

transport system and are concentrated fifteen to fifty times within alveolar macrophages in minutes (Johnson et al., 1980a; Hand and King-Thompson, 1982).

The ability of an antibiotic to enter the alveolar macrophage has been shown to correlate well with the efficacy of the drug in the treatment of certain intracellular pulmonary infections (Hand et al., 1984). This ability may also effect the host cell and the observation that tetracyclines have greater and more consistant effects on neutrophils than the B.lactams, may be a result of the former's greater uptake into the phagocytes.

Although clear conclusions and generalisations are difficult to make from the studies discussed, it would seem that many antibiotics at therapeutic concentrations may effect the hosts immune response, often adversely.

CHAPTER II

AIMS OF THE PRESENT STUDY

Pulmonary infection with mucoid, alginate producing P.aeruginosa is a major problem in CF. Studies discussed in the introduction have indicated that CF patients do not have a general immune defect, but that secondary immune defects may arise as a result of the infection. The advantages that the mucoid forms have over the non-mucoid forms were also discussed. The first aim of this thesis was to investigate the hypothesis that the production of alginate, gives mucoid P.aeruginosa a selective immunological advantage over non-mucoid forms.

The binding of bacteria to macrophages by specific receptors is the first step in the recognition process that leads to phagocytosis and the subsequent elimination of the bacteria. Therefore the effects of alginate isolated from a mucoid P.aeruginosa strain on rat alveolar macrophage function and receptor expression were investigated by studying:

1) The binding and phagocytosis of non-mucoid P.aeruginosa and Staph.albus.
2) The binding of antibody sensitised sheep erythrocytes to Fc receptors and the phagocytosis of latex.

The effect of alginate on the opsonisation of non-mucoid P.aeruginosa was also examined.

Mucoid P.aeruginosa are rarely eradicated from the CF lung even after intensive antibiotic therapy. Therefore, any adverse or advantageous effect an antibiotic may have on the alveolar macrophage, which as discussed previously, is so important in pulmonary defense, may argue against or in favour of its long term use. The second aim of this thesis was to investigate the effect of low levels of antibiotics (similar to those attained in the CF lung) on alveolar macrophage

function and receptor expression. Three antibiotics namely azlocillin, ticarcillin and tobramycin were studied at concentrations above and below the MIC for P.aeruginosa 492a Rev1. Monolayers of rat alveolar macrophages were prepared and assays were carried out to determine the effect of the antibiotics on:

1) Lectin-like receptors - by studying the binding of unopsonised bacteria.
2) Phagocytosis of opsonised P.aeruginosa.
3) Fc receptors by the rosette technique using antibody sensitised sheep erythrocytes.

The antibiotics were either pre-incubated with the macrophages or added to the monolayers with the indicator cells. For each assay the effect of the antibiotics on the indicator cells (opsonised bacteria or sensitised erythrocytes) was also examined.

The mechanism of action of tobramycin on Fc receptors was further investigated by studying the effect of supernatants taken from tobramycin-treated macrophages on:

1) The binding of EA_G to Fc receptors after treatment of the erythrocytes with supernatant.
2) The binding of EA_G to supernatant-treated alveolar macrophage monolayers.
3) The agglutination of EA_G.

Polyacrylamide gel electrophoresis was also carried out to determine the protein content of the supernatants.

Experiments on the effect of the three antibiotics on human monocyte Fc receptor expression were performed to determine the relevance of the animal studies to the human situation.

CHAPTER III

MATERIALS AND METHODS

1.0 BUFFERS AND STAINS

All chemicals were analytical grade, obtained from B.D.H. Chemicals Ltd., Poole, Dorset or Fisons Scientific apparatus, Loughborough, Leicestershire, unless otherwise stated.

1.1 Dulbecco's phosphate buffered saline (D.PBS)

This was prepared from tablets (Oxoid, Basingstoke). One tablet was dissolved in 100 ml of distilled water.

1.2 D.PBS with calcium and magnesium (D.PBS.'B')

This was prepared by dissolving 2g CaCl and 2g MgCl in 100 ml of distilled water. 0.5 ml was then added to 100 ml D.PBS.

1.3 Eagle's, Minimum Essential Medium (MEM)

Eagle's MEM was prepared by mixing 10 ml of 10 times concentrated Eagle's MEM (Wellcome Research Laboratories, Beckenham, Kent), 1 ml, 1M Hepes, 5 ml, 4% sodium bicarbonate, 1 ml, 0.2M glutamine and making the volume up to 100 ml with distilled water.

1.3.1 Eagle's MEM with antibiotics

This was prepared as above except that 0.1 ml of penicillin and streptomycin (200,000 U ml^{-1}) were added.

1.4 EDTA (20mM)

3.72g of EDTA were dissolved and made up to 500 ml with distilled water.

1.5 Formol-saline

0.5 ml of formaldehyde was added to 100 ml of 0.9% saline.

1.6 Glycine buffer

16.3g of aminoacetic acid (glycine) and 12.65g of NaCl were dissolved in distilled water. The pH was adjusted to 8.2 with NaOH (10% solution) and the mixture made up to 1 litre with distilled water.

1.7 Glycine, Bovine serum albumin (G.BSA)

One g of bovine serum albumin (Sigma*) was added to one litre of glycine buffer.

1.8 Hanks balanced salt solution (HBSS)

Hanks stock solution (Oxoid, Basingstoke) pack A and pack B were dissolved in one litre of distilled water. Equal volumes (50ml) of each stock solution were added together and made up to one litre with distilled water.

1.9 Formol-HBSS (1%)

One ml of formaldehyde was added to 99ml HBSS.

1.10 Gelatin-HBSS (1%)

One g of gelatin was dissolved in 100 ml of warm HBSS.

* Poole, Dorset

1.11 Hepes buffer

This was prepared by dissolving 23.83g Hepes buffer (Flow Laboratories, Irvine, Scotland) in distilled water. The pH was adjusted to 7.6 with 5N.NaOH and the final solution made up to 100 ml with distilled water.

1.12 Iso-sensitest broth

23.4g of iso-sensitest broth (Oxoid, Basingstoke) were dissolved and made up to one litre with distilled water.

1.13 Lysis buffer

This was prepared by dissolving 8.2g NH_4Cl, 1g $KHCO_3$ and 0.037g Na_2EDTA and making up to one litre with distilled water.

1.14 Sodium bicarbonate buffer (4%)

Five g of sodium bicarbonate were dissolved and made up to 100 ml with distilled water.

1.15 Sodium chloride (2.5M)

This was prepared by dissolving 14.6g of NaCl and making up to 100 ml with distilled water.

1.16 Tris HCl buffer (5mM)

This was prepared by dissolving 2.42g of Tris buffer (Tris hydroxymethyl methylamine) and making up to one litre with distilled water. The pH was adjusted to 7.4 with 1N HCl and the volume made up to four litres with distilled water.

1.17 May Grunwald/Giemsa stain

May Grunwald stain was diluted 1:6 with buffered water, pH 6.8 (tablets from Gurr, Searle Laboratories).

Giemsa stain was diluted 1:30 with buffered water. Cell monolayers were stained for three minutes in each stain in the above order, and washed with water.

1.18 Trypan blue

1% aqueous trypan blue was added to the cell suspension in a 1:1 ratio.

1.19 White cell diluting fluid

A stock solution was prepared by adding 0.1g gentian violet to 100ml of 1% acetic acid. For use, the stock was diluted 1:10 with 1% acetic acid and one part cell suspension was added to nine parts stain.

2.0 PURIFICATION OF CELLS

2.1 Animals

Male Sprague-Dawley, specific pathogen free (SPF) rats were obtained from the centre for laboratory animals, Edinburgh University.

2.2 Rat alveolar macrophages

Rats were killed with sodium pentobarbitone, (50mg/animal I.P.), exsanguinated and the trachea and lungs removed. Alveolar macrophages were obtained by lavage, via a tracheal cannula, with pre-warmed D.PBS and heparin (10U ml^{-1}). Fifty ml of lavage fluid was collected and centrifuged at 200g for 10 minutes. The macrophages were suspended in Eagle's MEM to give a final viable cell count of 2×10^5 ml^{-1}.

2.3 Human monocytes

Monocytes were separated according to the method of Boyum (1968). Blood from healthy donors was drawn into siliconised universals, containing 10 units of heparin ml^{-1} and diluted with an equal volume of physiological saline. The diluted blood was layered onto Ficoll-Hypaque cushions, specific gravity 1.078 (Pharmacia, Uppsala, Sweden) in a 3:1 ratio and centrifuged for 40 minutes at 400g. The cells at the interface containing monocytes and lymphocytes were collected, washed twice in D.PBS and resuspended in Eagle's MEM to give a final viable cell count of $10^6 ml^{-1}$.

2.4 Preparation of monolayers

One ml of the prepared cell suspension was layered onto 13mm diameter glass coverslips in 16mm well diameter tissue culture plates (Nunc, Copenhagen) and incubated for 30 minutes at 37°C. Non-adherent cells were removed by washing with warm Eagle's MEM.

3.0 PREPARATION OF THE INDICATOR CELLS

3.1 P.aeruginosa

P.aeruginosa strains were obtained from Dr J.R.W. Govan, Bacteriology Department, University of Edinburgh. Strain 492a is a mucoid, alginate-producing strain isolated from the sputum of a cystic fibrosis patient and 492a Rev1 is an isogenic non-mucoid revertant of this strain.

The bacteria were grown for 24 hours at 37°C on nutrient agar (Columbia agar base, Oxoid) and harvested by scraping from the plate with a glass slide. Killed suspensions were prepared (from viable suspensions) by 24 hour exposure to 0.5% formalin at 4°C, followed by washing with saline three times.

3.1.1 Optical density calibration of P.aeruginosa

To permit the rapid and consistant quantification of the number of bacteria in a suspension, a calibration curve based on optical density was constructed. Bacterial suspensions of P.aeruginosa 492a and 492a Rev1 were diluted and absorbancy measured on a spectrophotometer (UV30 Pye Unicam) set at a wave length of 540nm. Colony counts were then performed on the suspensions, so that a curve could be constructed (Figure 1). This curve was used to estimate the whole cell concentration of P.aeruginosa suspensions in all subsequent experiments involving the bacteria.

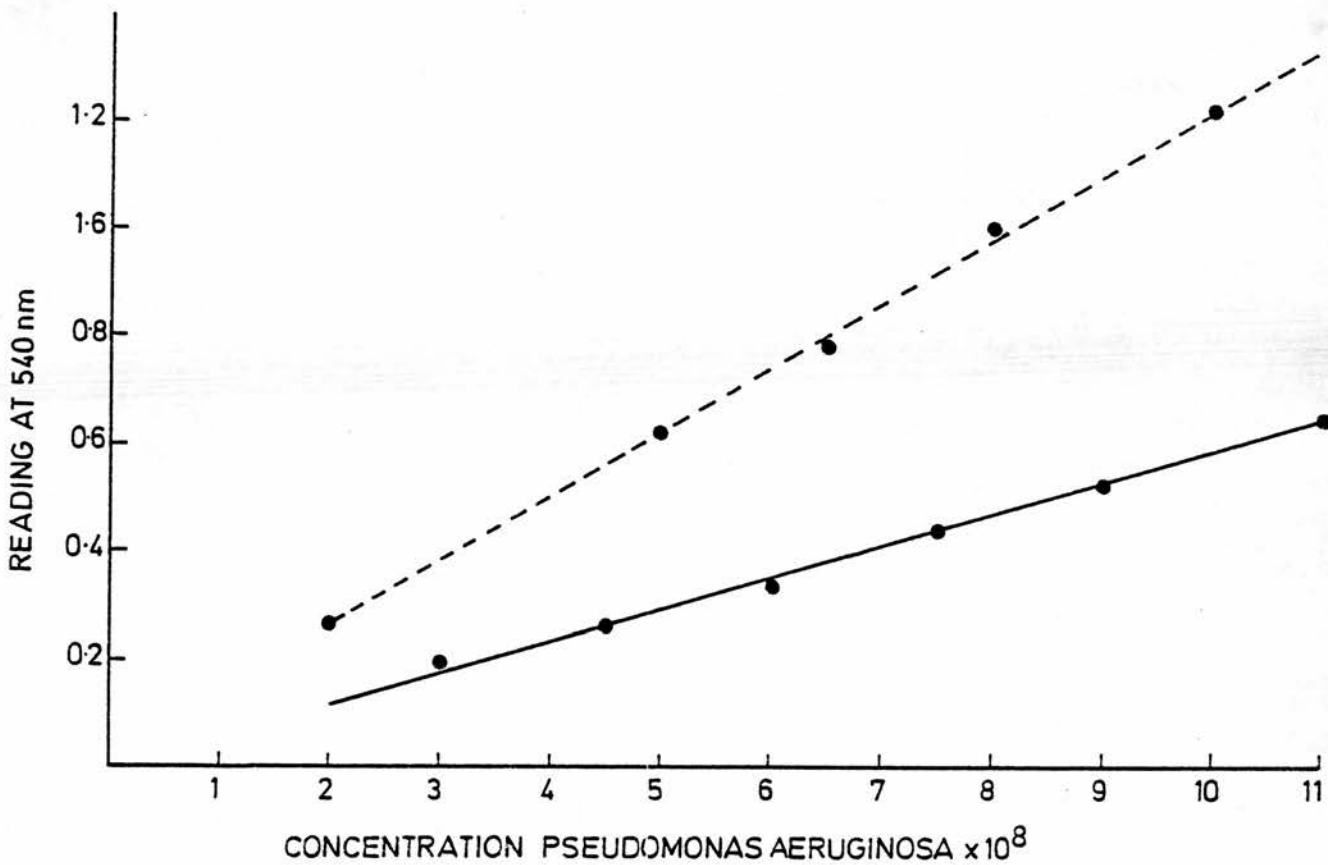


Figure 1

Optical density calibration curve of mucoid P.aeruginosa 492a and its nonmucoid revertant 492a Rev1.

(● - - - - ●) 492a (mucoid)

(● —— ●) 492a Rev1 (non-mucoid)

The y axis shows the absorbancy of the bacterial suspension at a wavelength of 540nM.

3.2 Staphylococcus albus

A strain of staph albus was obtained from the Departmental teaching collection. The organisms were grown in horse digest broth and harvested in log phase. Killed suspensions were prepared from viable suspensions by 24 hour exposure to 0.5% formalin at 4°C. Bacterial concentration was estimated spectrophotometrically at a wavelength of 540nm. An absorbancy of 0.42 was equivalent to 2×10^7 organisms ml^{-1}.

3.3. Preparation of immune serum

A suspension of heat-killed P.aeruginosa 492a Rev1 was washed and resuspended in saline at a concentration of $10^{10} ml^{-1}$. The bacteria were mixed with an equal volume of a 2% suspension of alhydrogel (Miles Laboratories, Slough) at 37°C for one hour. The mixture was then centrifuged at 2,000g for 15 minutes and the bacterial pellet resuspended in saline at a final concentration of $10^{10} ml^{-1}$. One ml was injected I.P. into each rat four times at weekly intervals. One week after the last injection the animals were killed and exsanguinated. Blood was collected from the posterior vena cava, allowed to clot and centrifuged at 1,000g for 30 minutes. The serum was heat-inactivated (56°C for 30 minutes) and stored at -20°C.

3.4 Detection of agglutinating antibody

A suspension of P.aeruginosa 492a Rev1 ($10^{10} ml^{-1}$) was prepared in G.BSA. Latex suspension (Difco) was adjusted spetrophotometrically to read 192 at 540nm and a 0.5% solution prepared in G.BSA. The

bacteria and latex solutions were mixed in equal volumes and left at room temperature for five minutes. Doubling dilutions of immune serum were made in a V-bottomed micro titre plate (Sterilin) so that each well contained 25ul of serum and 25ul of the bacteria and latex mixture was added to each well. The plate was incubated for two hours at 37°C and left overnight at 4°C. Streaming of the latex particles when the plate was tilted indicated that agglutination had not occurred.

3.5 Bacterial opsonisation

3.51 P.aeruginosa

Bacteria at a concentration of 10^9 ml^{-1} were added to immune serum diluted with G.HBSS in a 1:10 ratio. The suspension was mixed for 20 minutes at 37°C and centrifuged at 2,000g for 15 minutes. The bacterial pellet was resuspended in HBSS to give a final concentration of 10^8 bacteria ml^{-1}.

3.5.2 Staph albus

Bacteria at a concentration of 5×10^8 ml^{-1} were added to h eat inactivated normal rat serum diluted in G.HBSS in a 1:10 ratio. The procedure was continued as above and the bacteria resuspended in HBSS to give a final concentration of 5×10^7 ml^{-1}.

3.6 Preparation of IgG2b sensitised sheep red blood cells (EA_G)

Sheep cells in alsevers were obtained from tissue culture services (Slough, Berkshire). The red cells were washed three times with

HBSS and suspended at a final concentration of $10^9 ml^{-1}$. This concentration was determined by making a 1:30 dilution of red cells with distilled water and allowing the cells to lyse. At a red cell concentration of $10^9 ml^{-1}$, this solution will read 0.35 at 541nm on a spectrophotometer (Pye Unicam, UV30).

An equal volume of antibody, in this case a mouse monoclonal IgG2b against sheep red cells (Clone Sp2HL, Sera Laboratories, Crawley Down) was mixed with sheep red blood cells ($10^9 ml^{-1}$) and incubated at 37°C for 30 minutes. The red cells were then washed three times in HBSS and resuspended to a final cell concentration of $2.5 \times 10^7 ml^{-1}$.

3.7 Formolisation of red blood cells

Sheep red blood cells were washed three times with D.PBS resuspended in 100 ml of D.PBS for every 20 ml of packed cells and placed in a conical flask. 20 ml of formalin was added to visking tubing submerged in the red cell suspension and gently stirred at 4°C for 3 hours. The swollen dialysis sac was then punctured, allowing the formalin to slowly escape, and removed. Gentle mixing was then continued for 18 hours (overnight). The formalised cells were then washed five times with D.PBS and stored at 4°C. Prior to use they were washed twice in HBSS. The erythrocytes were sensitised with antibody as described above.

3.8 Preparation of complement coated sheep red blood cells(EAc)

An equal volume of sheep red blood cells ($10^9 ml^{-1}$) were mixed with a mouse monoclonal IgM (Clone Sp1 HL, Sera-Lab, Crawley Down)

at the maximum sub-agglutinating dose, in this case a 1:5 dilution. The mixture was incubated at 37°C for 30 minutes, washed twice in HBSS and resuspended in HBSS at a final cell concentration of $10^8 ml^{-1}$, to give EA_M.

A preparation of yeast cell walls in azide 0.2ml (obtained from Dr J Stewart, Department of Bacteriology) was washed twice and suspended in 1ml HBSS. One ml of AB serum (previously absorbed against sheep red blood cells) was added and the mixture incubated at 37°C for 30 minutes and centrifuged at 1,000g for 10 minutes. The supernate (R_3) was collected and serially diluted. Equal volumes of R_3 and EA_M ($10^8 ml^{-1}$) were incubated at 37°C for 40 minutes. The mixture was washed twice and resuspended to a final cell concentration of $2.5 \times 10^7 ml^{-1}$.

4. ASSAYS

At the end of each assay described in this section, the coverslips were air-dried, fixed in methanol and stained with May Grunwald/Giemsa. Triplicate coverslips were used and 200 macrophages counted on each coverslip.

4.1 Bacterial binding

Macrophage monolayers were overlaid with one ml of Staph.albus (5×10^7 ml^{-1}) or P.aeruginosa ($5 \times 10^8 ml^{-1}$) in D.PBS.'B' and incubated for two hours at $4^{\circ}C$. The monolayers were then washed ten times with D.PBS'B'. The results were expressed as the percentage of cells binding bacteria at two or more discrete points.

4.2 Phagocytosis

One ml of opsonised bacteria were overlaid onto macrophage monolayers and incubated for 30 minutes at $37^{\circ}C$. Extracellular bacteria were removed by vigorous washing with HBSS (ten times). The results were expressed as the percentage of macrophages that contained five or more bacteria.

4.3 Fc receptor assay

Two ml of EA_G (2.5×10^7 ml^{-1}) were added to the macrophage or monocyte monolayer centrifuged for four minutes at 30g and incubated for 30 minutes at $22^{\circ}C$ (room temperature). Non-adherent red cells were removed by gentle washing with HBSS and the coverslips were finally dipped into 1% formol HBSS to prevent red cell lysis.

The results were expressed as the percentage of macrophages binding two or more erythrocytes.

4.4 Phagocytosis of EA_G

Two ml of EA_G (2.5×10^7 ml^{-1}) were added to the macrophage monolayer, centrifuged for four minutes at 30g and incubated for 30 minutes at 37°C. Extracellular erythrocytes were removed by washing five times with HBSS.

The results were expressed as the percentage of macrophages that had phagocytosed two or more erythrocytes.

4.5 Phagocytosis of latex

The macrophage monolayers were overlaid with one ml of HBSS and 20ul of latex suspension (Difco Laboratories, Surrey) adjusted spectrophotometrically to read 192 at a wavelength of 540nm. The monolayers were then incubated for one hour at 37°C. Non-phagocytosed particles were removed by repeated washing with HBSS.

The results were expressed as the percentage of cells that had taken up five or more latex particles.

4.6 C3 receptor assay

Two ml of EAc ($2.5 \times 10^7 ml^{-1}$) were added to the macrophage monolayer and the procedure carried out as previously described for the detection of Fc receptors.

5.0 ALGINATE STUDIES

5.1 Production and extraction of Pseudomonas alginate

The method used was a modification of that reported by Evans and Linker (1973) and Govan and Fyfe (1978). Colonies of P.aeruginosa 492a were taken from 24 hour culture grown on Pseudomonas Isolation agar (PIA, Difco) at 37°C and used to inoculate 30 ml volumes of broth containing 1% Bacto-peptone (Difco) and 2% sodium gluconate. The cultures were grown for 48 hours in an orbital incubator 100 rev min^{-1} at 37°C to achieve maximum alginate production. The culture broth was mixed with 2.5M NaCl in a ratio of 25:1 and centrifuged at 20,000g for one hour. The bacterial pellet was discarded and the supernatant spun for one hour at 20,000g. Three volumes of 95% ethanol were slowly added to the supernate and gently stirred. The resultant gelatinous precipitate was removed by means of a glass rod, washed twice in 95% ethanol, once with absolute ethanol,freeze-dried and stored at 4°C. The alginate was checked for contamination with protein and nucleic acid by measuring spectrophotometric extinction values at 280 and 260nm.

5.2 Treatment of macrophages with alginate

Alginate was reconstituted in HBSS at concentrations ranging from 0.25 mg ml^{-1} to 2mg ml^{-1}. One ml of alginate was added to macrophage monolayers and incubated at 37°C for 30 minutes. The macrophages were subsequently washed and the assays performed as described above. In some experiments vigorous washing was carried out and this involved washing the monolayers five times with HBSS

after the 30 minute incubation period.

5.3 Addition of alginate with the indicator cells

The alginate was reconstituted in HBSS (for red cells) or D.PBS'B' (for bacterial binding) and one ml was added to the monolayers with the EA_G or bacteria.

6.0 ANTIBIOTIC STUDIES

The three antibiotics used in this study were:

Tobramycin - Eli Lilly, Basingstoke, England

Azlocillin - Bayer, Germany

Ticarcillin - Beecham, Brentford, England

6.1 Determination of the minimum inhibitory concentration (MIC)

The stock antibiotic was diluted in sterile distilled water to ten times the highest test concentration, then finally diluted in isosensitest broth. Doubling dilutions were made in sterile glass tubes. For each set of dilutions, there was a growth control (no antibiotic) and a media control (no inoculum). Each tube (except the media control) was inoculated with 100ul of P.aeruginosa 492a Rev1 at $10^5 ml^{-1}$ in isosensitest broth and incubated for 24 hours at 37^oC. The growth of bacteria was monitored by the turbidity of the suspension. The MIC was taken as the lowest concentration of antibiotic at which there was no turbidity in the overnight culture. Solutions were checked by plating out 0.1 ml on nutrient agar and measuring growth after 18 hours. Calcium was added to the isosensitest broth to give a final concentration of 1.6mM, to allow for the calcium concentration of the media used in the assays.

6.2 Antibiotic treatment of macrophages

a) The antibiotics were diluted in Eagle's MEM and one ml was added to the monolayers. The macrophages were then incubated at 37^oC and washed once prior to performing the assays

described above.

b) The antibiotics were diluted in HBSS and added to the macrophage monolayer with the indicator cells.

6.3 Treatment of indicator cells with antibiotics

One ml of EA_G ($10^8 ml^{-1}$) or opsonised bacteria (5×10^8 ml^{-1}) were incubated with an equal volume of antibiotic dilutions in HBSS for 30 minutes at 37°C. The mixture was then centrifuged at 200g and the erythrocyte or bacterial pellet washed twice with HBSS. The cells were resuspended in HBSS and an Fc receptor or phagocytic assay performed as described above.

7.0 STUDIES ON THE SUPERNATANTS

7.1 Preparation of dialysis tubing

Dialysis tubing, $^{8}/_{32}$, (Scientific Instrument Centre, London) was boiled in 2% $NaHCO_3$ for one hour, washed and boiled in 20mM EDTA for one hour. The tubing was rinsed thoroughly in distilled water and stored at 4°C in 20mM EDTA. Prior to use the tubing was washed with distilled water.

7.2 Preparation of supernatants

Rat alveolar macrophage monolayers were incubated with tobramycin diluted in Eagle's MEM at 37°C. After gentle shaking, the supernatants were removed and frozen at -20°C. The frozen material was then freeze-dried, reconstituted in a small amount of distilled water and dialysed against 5mM Tris HCl for 48 hours at 4°C. When dialysis was complete, the dialysate appeared clear and was removed from the tubing. The solutions were freeze dried and stored at -20°C.

7.3 Supernatant inhibition studies

7.3.1 EA_G

The freeze dried supernatants were reconstituted to their original volume with HBSS and incubated with an equal volume of EA_G ($10^8 ml^{-1}$) for 30 minutes at 37°C. The erythrocytes were then washed twice in HBSS and resuspended at 2.5×10^7 ml^{-1}. The Fc receptor assay was then performed as previously described.

7.3.2 Macrophages

The freeze dried supernatants were reconstituted to the original volume in HBSS and incubated with macrophage monolayers at 22°C for one hour. The monolayers were then washed twice and the Fc receptor assay performed as previously described.

7.4 Polyacrylamide gel electrophoresis (PAGE)

PAGE was performed in the presence of sodium dodecyl sulphate (SDS) on 10% (w/v) polyacrylamide slab gels with a 10mM 4% (w/v) stacking gel as described by Poxton and Brown (1979). The discontinuous buffer system (gels at different pH) of Laemelli (1970) was used. Freeze dried supernatants were reconstituted in 100ul of single strength sample buffer and treated for three minutes in a boiling water bath. 60 ul of each sample was loaded onto the gel. Electrophoresis was performed at constant voltage, initially at 60 V, until the sample had reached the separating gel, then at 150 V until the bromophenol blue tracking dye was approximately 5 cm from the bottom of the gel. The gel was carefully removed from the glass plates and stained overnight with Coomassie Brilliant Blue G or R (Sigma) and destained with the solutions described by Poxton and Sutherland (1976) to identify the protein bands.

The molecular weight marker used in these studies was in the range 12,300 - 78,000 daltons.

7.5 Agglutination of erythrocytes

EA_G were prepared as previously described ($2 \times 10^8 ml^{-1}$), supernatants were serially diluted in a V bottomed 96 well plate (Sterilin) so that each well contained 50ul. EA_G were added (50ul) mixed and incubated for one hour at room temperature, to allow agglutination to occur.

7.6 Inhibition of agglutination

Supernatants were incubated with formalised EA_G for two hours at $4^{\circ}C$. The mixture was centrifuged at 400g for 10 minutes, the supernatants collected and tested for EA_G agglutination as above.

8.0 CALCULATIONS

The percentage inhibition or enhancement was calculated as the difference between control and test divided by the control times 100.

$$\text{Inhibition} = \frac{C - T}{C} \times 100$$

$$\text{Enhancement} = \frac{T - C}{C} \times 100$$

8.1 Statistics

Analysis was performed by the student 't' test. As differences between experiments were similar to differences within an experiment, the means from experiments performed on different days were pooled.

CHAPTER IV

RESULTS

PART A

THE EFFECT OF PSEUDOMONAS ALGINATE ON RAT ALVEOLAR MACROPHAGES.

INTRODUCTION

The establishment of mucoid, alginate producing strains of P.aeruginosa in the lungs of CF patients is associated with a poor prognosis (Henry et al., 1982) and contributes to the persistence of the infection (Doring and Høiby, 1983). This suggests that the bacterial alginate may be a contributing factor in the pathogenesis of Pseudomonas infection in CF patients.

The immune status of the CF patient was discussed in the Introduction. Failure to eradicate mucoid P.aeruginosa from the CF lung is not thought to be due to a general immune defect (di Sant'Agnese and Davis, 1976). However it is possible that immune function in the CF lung may be impaired and the infection exacerbated as a result of alginate production by P.aeruginosa.

The purpose of these studies was to investigate if alginate had any effects on alveolar macrophages in the hope of furthering the understanding of the pathogenic properties of alginate. Alginate separated from a mucoid strain of P.aeruginosa was used to determine the effect on phagocytosis and binding of particles to rat alveolar macrophages. Preliminary experiments were necessary to determine the optimal conditions for demonstrating the presence of receptors and the phagocytosis of opsonised bacteria. These experiments are described at the beginning of the relevant section.

SECTION I

THE EFFECT OF ALGINATE ON "LECTIN-LIKE" RECEPTORS

1.0 DETERMINATION OF OPTIMAL CONDITIONS FOR THE BINDING OF P.AERUGINOSA AND STAPH.ALBUS TO RAT ALVEOLAR MACROPHAGES

Optimal conditions for bacterial binding to C_3H mouse peritoneal macrophages has been determined by H.Ogmundsdöttir (PhD thesis, 1979). She showed that binding of bacteria to macrophage monolayers took place at $4^{\circ}C$ and required two hours incubation for maximal binding. These conditions were shown to apply to a number of bacterial species including Staphylococcus, Streptococcus, Escherichia and Pseudomonas (Freimer et al., 1978) and other cell types, including mouse alveolar macrophages (Glass et al., 1981).

Dose response curves for the binding of P.aeruginosa to rat alveolar macrophages were carried out under these conditions as shown in Figure 2. The percentage of macrophages binding bacteria was directly related to the bacterial concentration used. At concentrations of P.aeruginosa 492a Rev1 above 10^{10} ml^{-1}, the number of background bacteria made the preparation difficult to count. At concentrations of Staph.albus above 10^{9} ml^{-1} no further increase in binding was observed. Formalin-killed P.aeruginosa 492a Rev1 were more readily bound than live bacteria, although less readily bound than Staph.albus.

Unwashed preparations of mucoid P.aeruginosa 492a did not bind to rat alveolar macrophages, even at concentrations of 10^{11} organisms ml^{-1} where in contrast to P.aeruginosa 492a Rev1 the background was low. A similar effect had previously been observed for mouse peritoneal and pulmonary macrophages (Oliver and Weir, 1983).

Binding of Staph.albus subsequently led to phagocytosis if the monolayers were incubated at $37^{\circ}C$, but this was not the case for P.aeruginosa that remained attached without being phagocytosed.

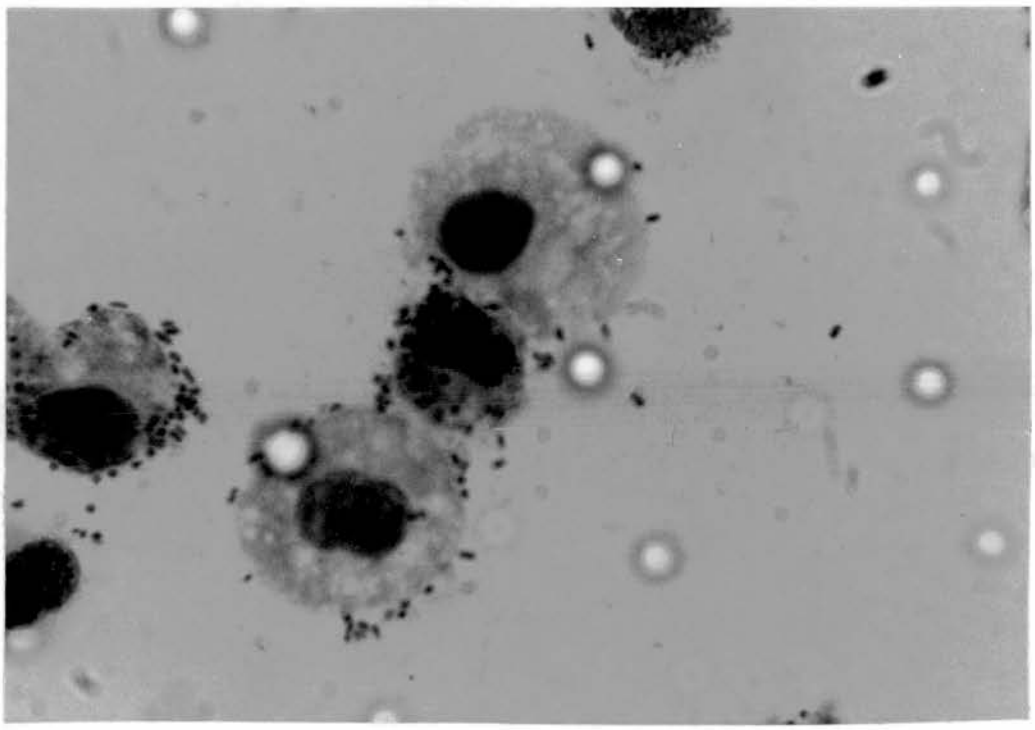


PLATE I

Binding of P.aeruginosa 492a Rev1 to rat alveolar macrophage monolayer (May Grunwald/Giemsa, x 1,250 magnification.)

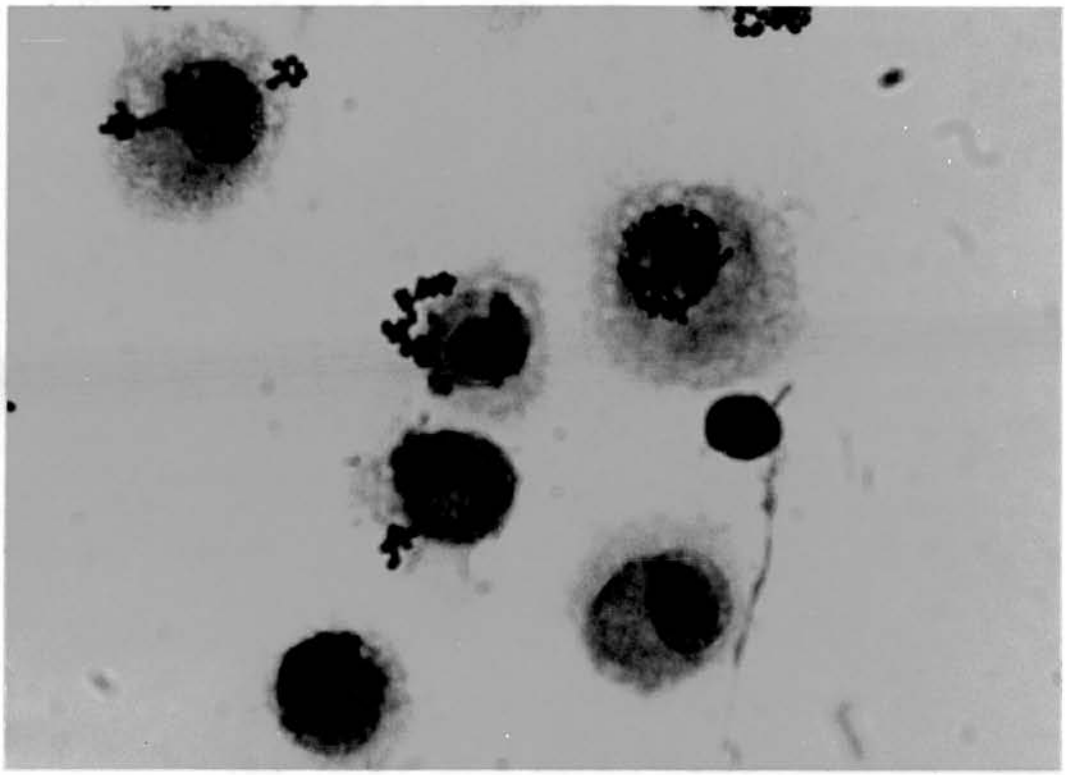


PLATE II

Binding of Staph.albus to rat alveolar macrophage monolayers (May Grunwald/Giemsa, x 1,250 magnification)

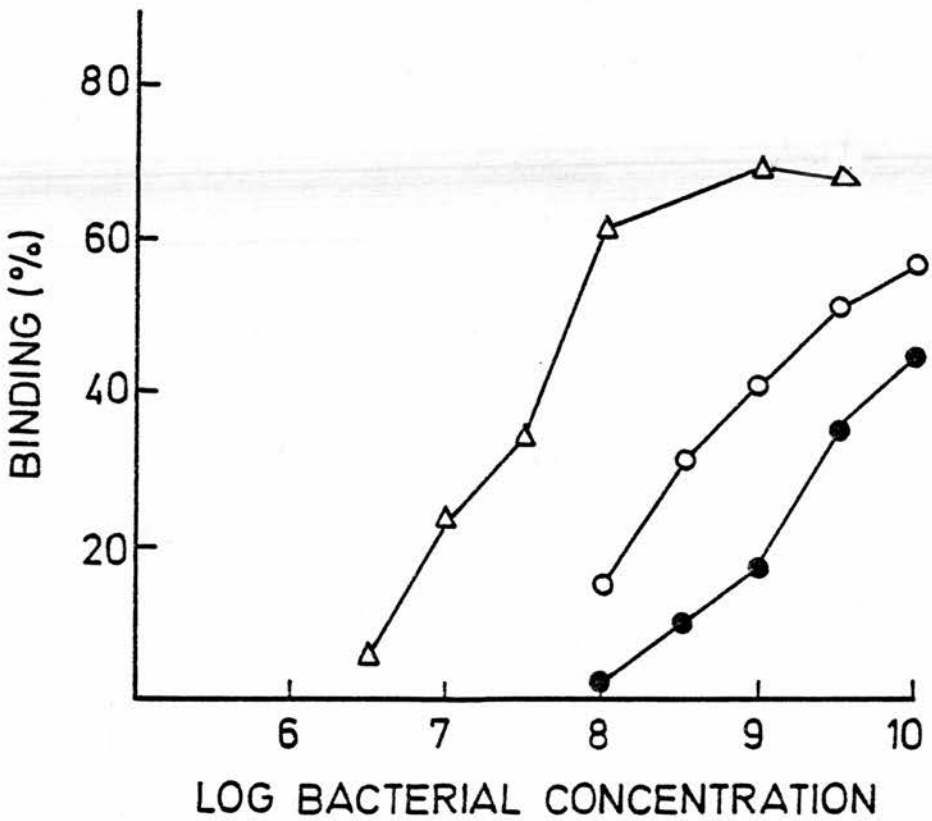


Figure 2

The effect of increasing concentrations of Staph.albus (△—△), formalin-killed P.aeruginosa 492 Rev1 (O—O) and live 492a Rev1 (●—●) on the binding by rat alveolar macrophages. Each point represents the mean of 2 experiments.

2.0 INHIBITION OF P.AERUGINOSA BINDING BY D-GLUCOSE AND D-GALACTOSE TO RAT ALVEOLAR MACROPHAGES.

Inhibition studies were carried out to determine whether P.aeruginosa bound to alveolar macrophages by the lectin-like receptor that had previously been identified on mouse alveolar macrophages for Staph.albus (Glass et al., 1981).

A suspension of live, washed P.aeruginosa was used at a concentration that gave 20-30% binding (2×10^9 ml^{-1}). Macrophage monolayers were pre-exposed to a range of concentrations of D-glucose and D-galactose (5-20 mM) prior to overlaying the P.aeruginosa as described in the Materials and Methods section. D-glucose and D-galactose reduced the binding of P.aeruginosa at 5, 10 and 20 mm concentrations ($P < 0.05$). Inhibition of binding reached a maximum at 10mm and the inhibition observed with glucose was slightly though not significantly greater than that observed with D-galactose, (Fig. 3.)

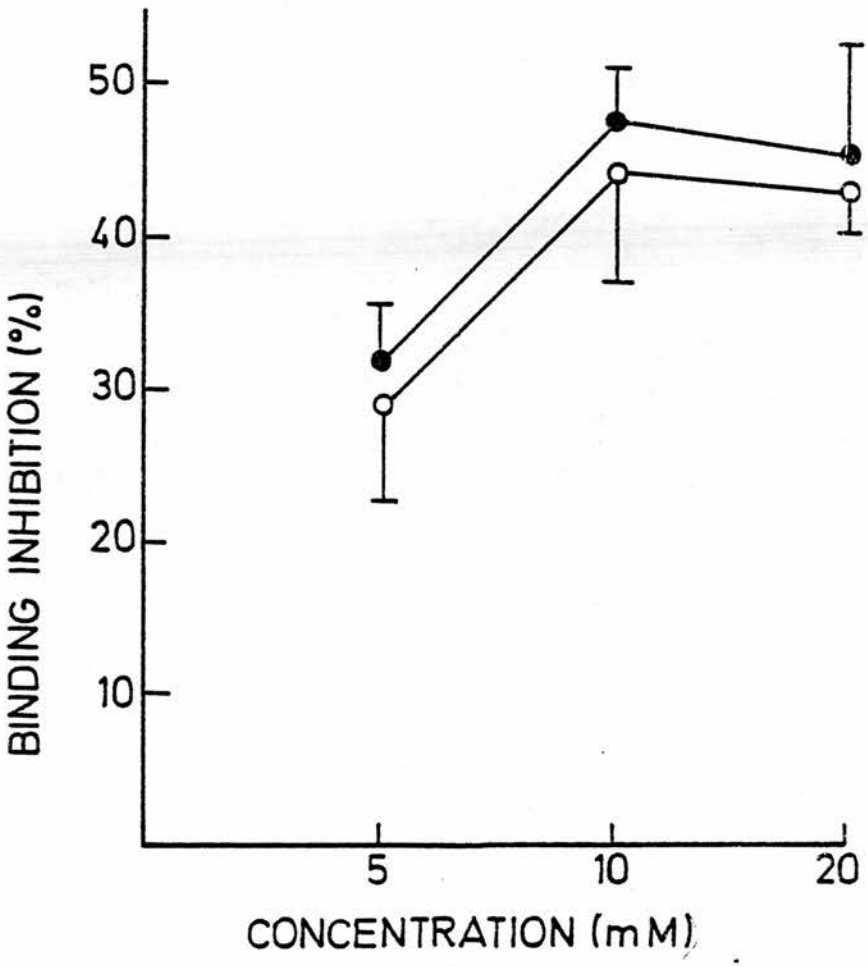


Figure 3

The effect of D-glucose (●——●) and D-galactose (O——O) on the binding of P.aeruginosa 492a Rev1 to rat alveolar macrophages. Each point represents the mean ± 1 SEM of three experiments.

3.0 THE EFFECT OF ALGINATE ON BACTERIAL BINDING TO RAT ALVEOLAR MACROPHAGES

The effect of increasing concentrations of pseudomonas alginate, extracted as described in the Materials and Methods section on the binding of a non-alginate producing P.aeruginosa and Staph.albus is shown in Figure 4. Concentrations of alginate above 2 mg ml^{-1} were not used, as at this viscosity of the material, it was difficult to ensure an even suspension over the monolayers.

The alginate obtained from strain 492a inhibited the binding of its non-mucoid variant 492a Rev1 to alveolar macrophages in a dose-dependent manner ($P < 0.05$). Similar results were obtained when the bacterial binding was studied using Staph.albus. When the macrophages were pre-incubated with alginate for 30 minutes, inhibition of the binding of Staph.albus was greater than that observed with P.aeruginosa reaching 70% at an alginate concentration of 2 mg ml^{-1}. Addition of the alginate to the monolayers at the same time as P.aeruginosa 492a Rev1, resulted in inhibition of binding that was greater ($P < 0.05$) than that observed when the macrophages were pre-treated with alginate.

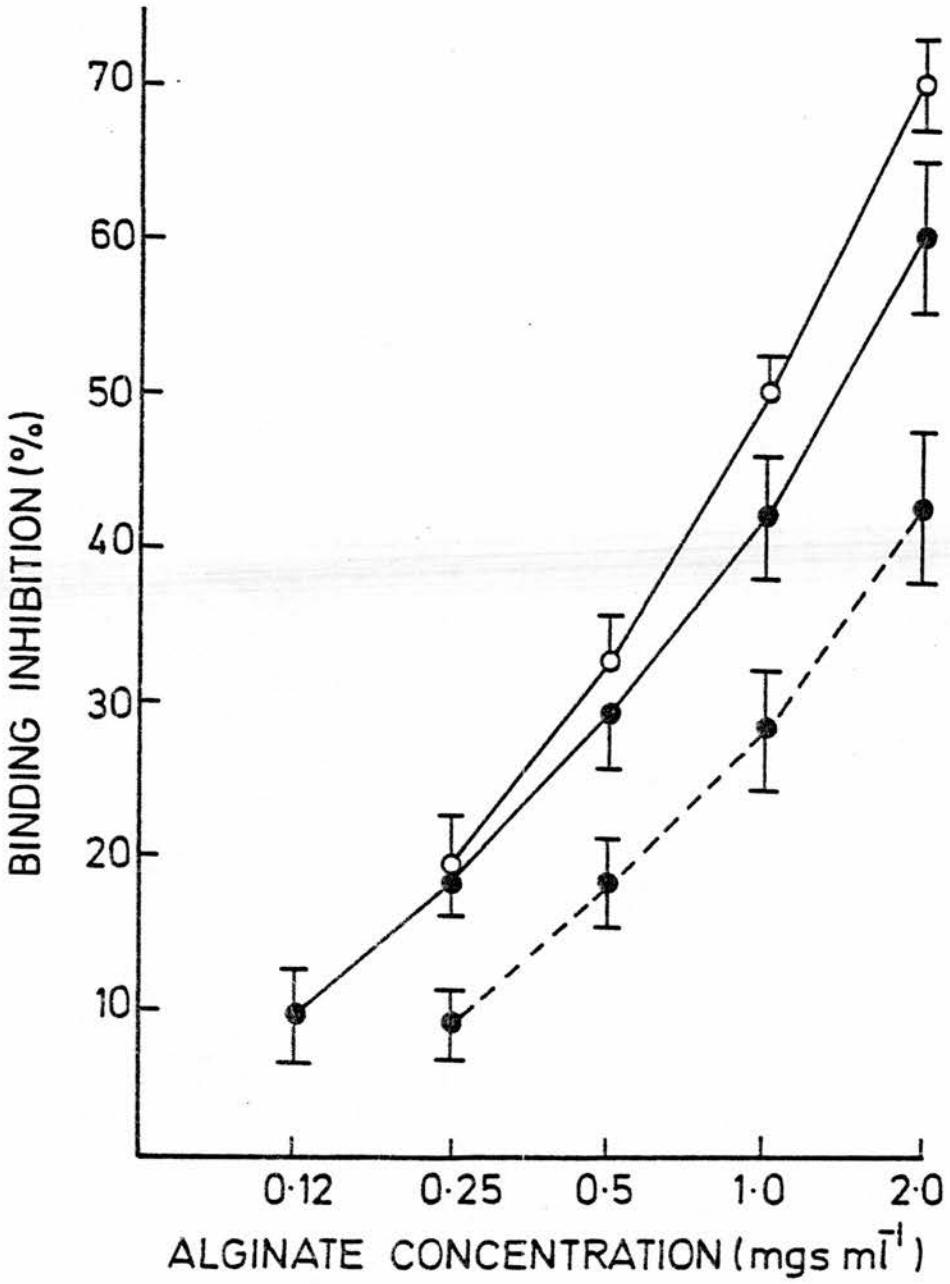


Figure 4

The effect of alginate on the binding of P.aeruginosa and Staph.albus to rat alveolar macrophages. Monolayers were pre-incubated with alginate for 30 minutes and P.aeruginosa (●---●) or Staph.albus (O——O) added. Alternatively alginate was added with P.aeruginosa to the monolayer (●——●). Each point represents the mean ± 1 SEM of four experiments.

4.0 SUMMARY

Staph.albus and a non-mucoid strain of P.aeruginosa bound in a dose-dependent fashion to rat alveolar macrophages. Binding was inhibited by D-glucose and D-galactose, indicating the involvement of the "lectin-like" receptor on these macrophages. Mucoid P.aeruginosa did not bind to the macrophages even at high concentrations of bacteria.

Alginate extracted from a mucoid strain of P.aeruginosa inhibited the binding of Staph.albus and P.aeruginosa to the macrophages in a dose-dependent fashion. This inhibition occurred if the cells were incubated with alginate prior to addition of the bacteria and in the case of P.aeruginosa the inhibition of binding was significantly increased when the alginate was added with the bacteria to the macrophage monolayers.

SECTION II

THE EFFECT OF ALGINATE ON PHAGOCYTOSIS

1.0 INTRODUCTION

As alginate inhibited the binding of P.aeruginosa it was considered important to determine its effect on the phagocytosis of opsonised bacteria, as unopsonised bacteria, as previously noted, were not taken up by these particular macrophages. It has been shown that pseudomonas alginate inhibits the phagocytosis of non-mucoid forms of the organism by rabbit neutrophils (Schwartzman and Boring, 1971). The purpose of these experiments was to extend these observations to a population of alveolar macrophages and investigate the effect of pre-incubation of these cells with alginate.

2.0 DETERMINATION OF THE OPTIMAL CONDITIONS FOR PHAGOCYTOSIS OF P.AERUGINOSA BY RAT ALVEOLAR MACROPHAGES

The strain of P.aeruginosa used in these studies was a non-mucoid revertant of the mucoid strain 492a, as described in Materials and Methods. Studies were not carried out with the mucoid bacterial strain for two reasons. First, opsonisation of these bacteria would necessitate a washing procedure, that would render the bacteria no longer mucoid, second, unopsonised bacteria were not phagocytosed and were toxic to the macrophages at concentrations above $10^8 ml^{-1}$.

For phagocytosis of P.aeruginosa 492a Rev1 by rat alveolar macrophages to occur, the bacteria were opsonised with specific immune serum. Opsonisation of the bacteria with fresh autologous serum, mouse serum, serum from rats intra-tracheally infected with P.aeruginosa, foetal calf serum or human serum did not result in phagocytosis of the bacteria by these cells. An immune serum was prepared as described in Materials and Methods. The serum was non-agglutinating as measured by the method described in Chapter III.

Dose response curves for the dilution of serum and bacterial concentration required for opsonisation and subsequent phagocytosis were constructed. The percentage of macrophages that had taken up bacteria was directly related to the dilution of antibody, although it reached a maximum at a dilution of 1/20 (Figure 5). Phagocytosis by the macrophages was also related to the concentration of bacteria in the opsonisation mixture and increased with increasing bacterial concentrations until a maximum was reached at 2×10^9 organisms ml^{-1} (Figure 6).

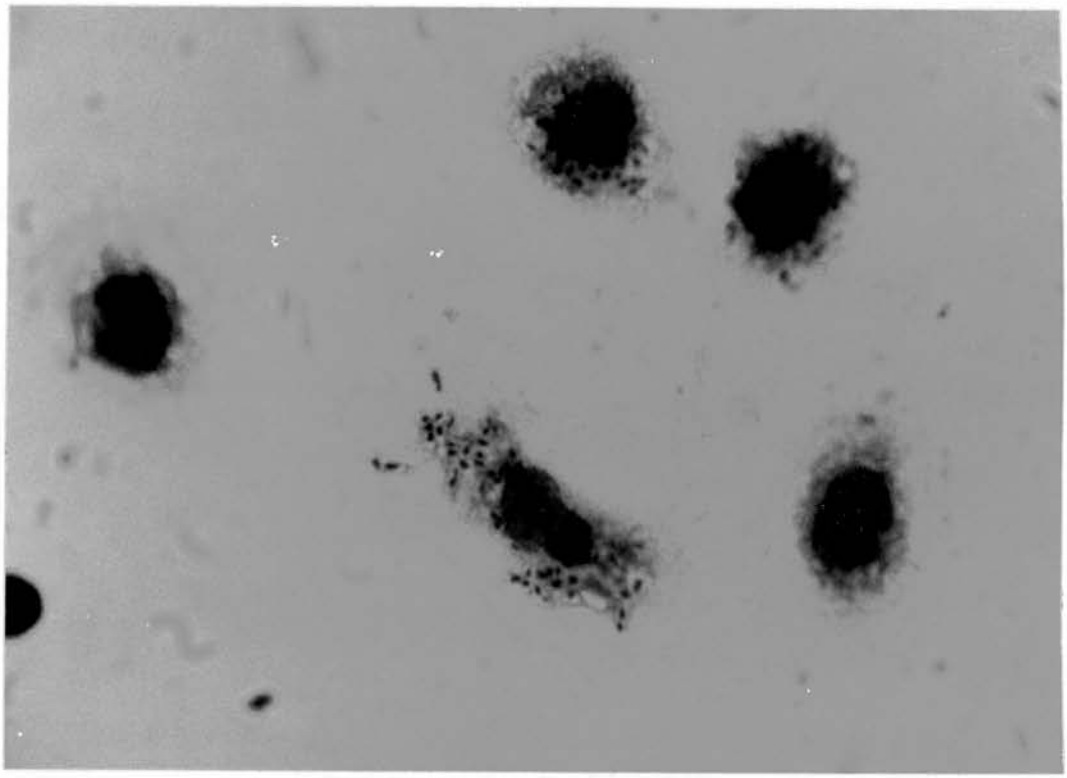


PLATE III

Phagocytosis of opsonised P.aeruginosa 492a Rev1 by rat alveolar macrophages (May Grunwald/Giemsa, x 1,250 magnification.)

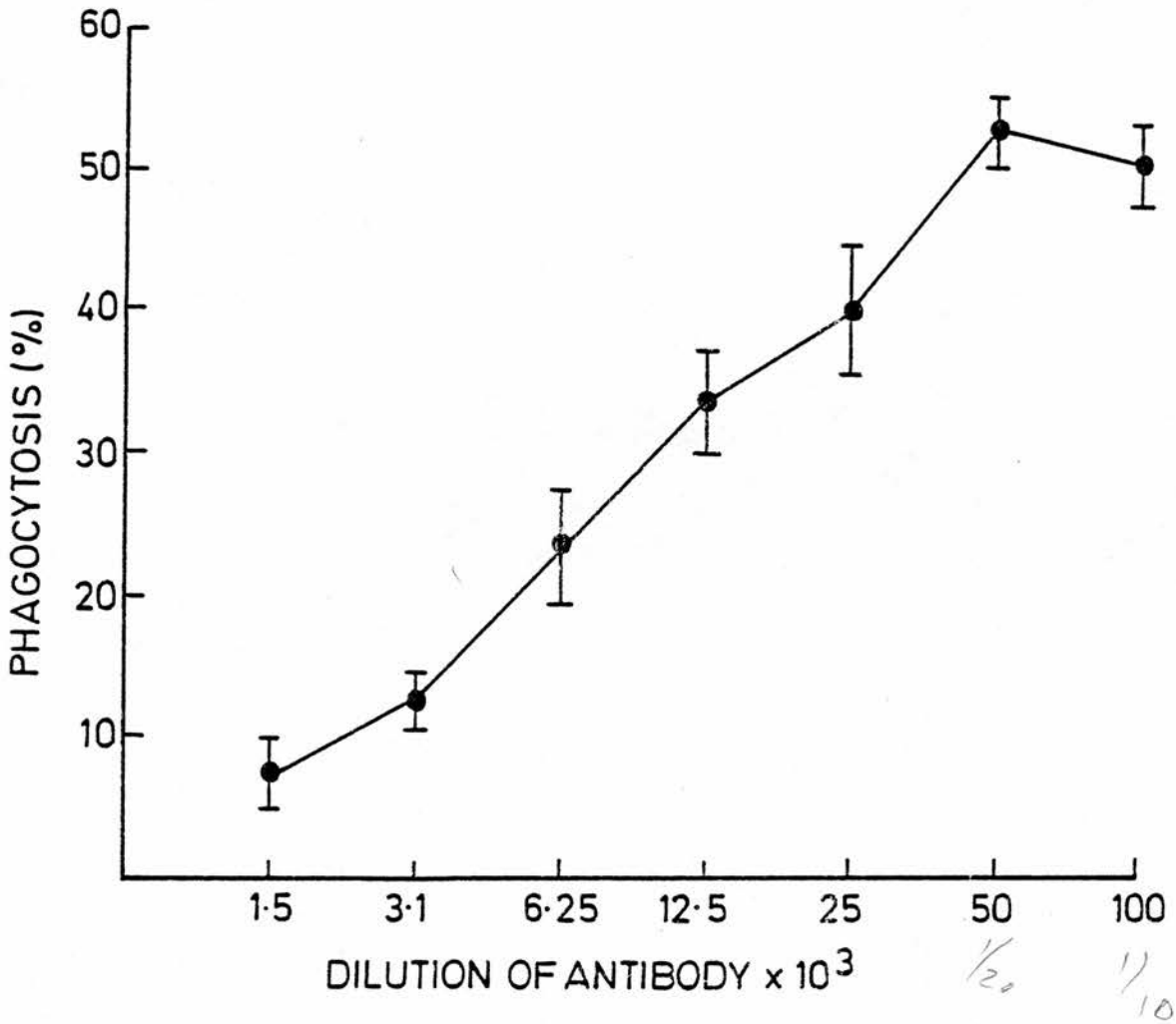


Figure 5

The effect of increasing the concentration of immune serum for opsonisation of P.aeruginosa on the phagocytosis of this organism by rat alveolar macrophages. Each point represents the mean ± 1 SEM of three experiments.

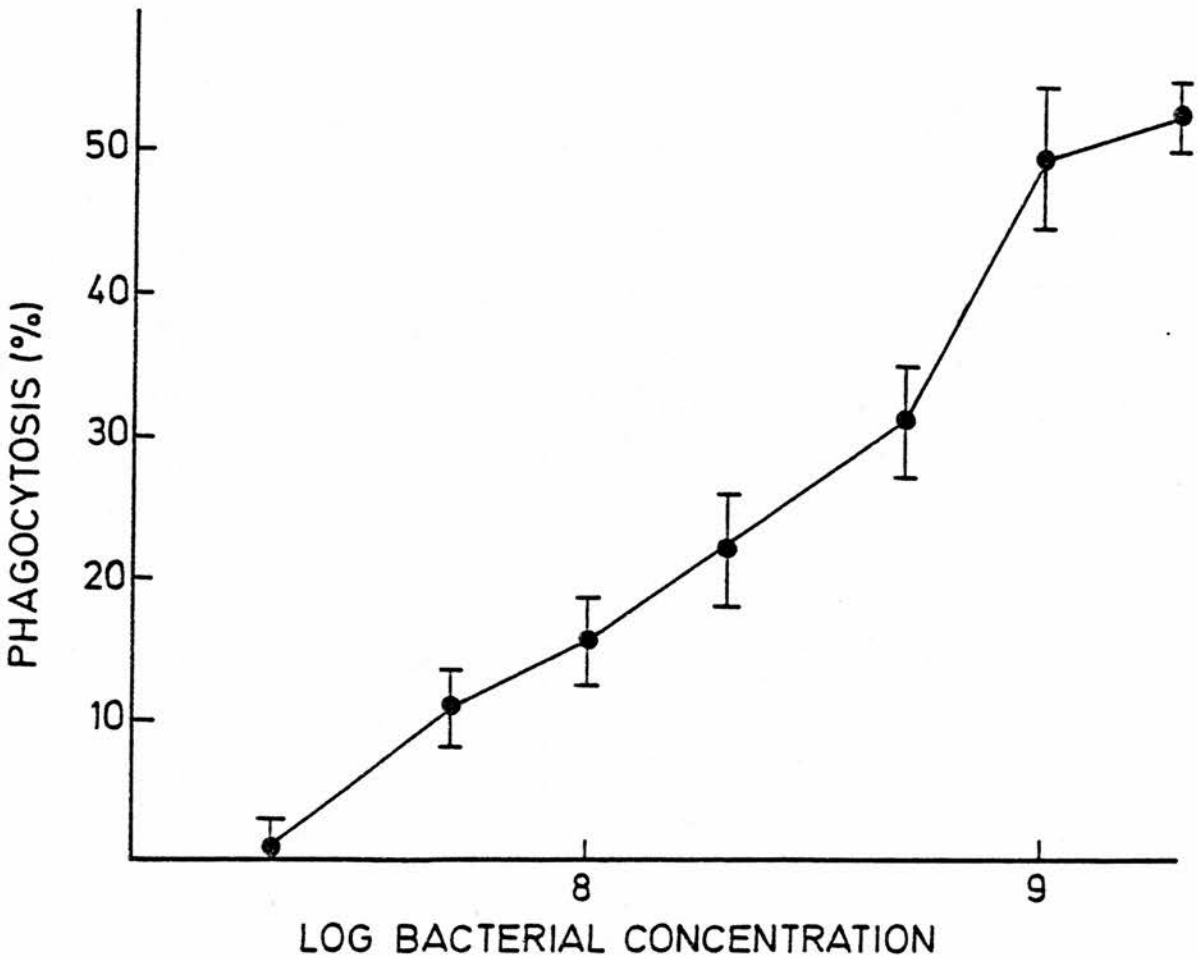


Figure 6

The effect on phagocytosis of increasing the concentration of *P. aeruginosa* in the opsonisation mixture, when a serum dilution of 1/20 was used. Each point represents the mean ± 1 SEM of three experiments.

Staph.albus was opsonised with heat-inactivated normal rat serum (presumably because of the presence of anti-staphlococcal antibody).

3.0 THE EFFECT OF ALGINATE ON THE PHAGOCYTOSIS OF P.AERUGINOSA

Bacteria were used at a final concentration of $5 \times 10^8 ml^{-1}$ with a 1/80 dilution of immune serum in the opsonisation mixture to give 30 - 40% phagocytosis.

Pseudomonas alginate inhibited the phagocytosis ($P < 0.05$) of non-mucoid P.aeruginosa when pre-incubated with the macrophage monolayers for 30 minutes. This effect was dependent on the concentration of alginate and was reduced by 50% if the macrophages were vigorously washed five times after alginate treatment (Figure 7). The phagocytic capacity of the macrophages returned to normal when these macrophages were re-incubated in Eagle's MEM alone for 30 minutes after alginate treatment.

Inhibition of phagocytosis reached a maximum of 90% when the alginate was added to the macrophage monolayers together with the opsonised bacteria (Figure 7).

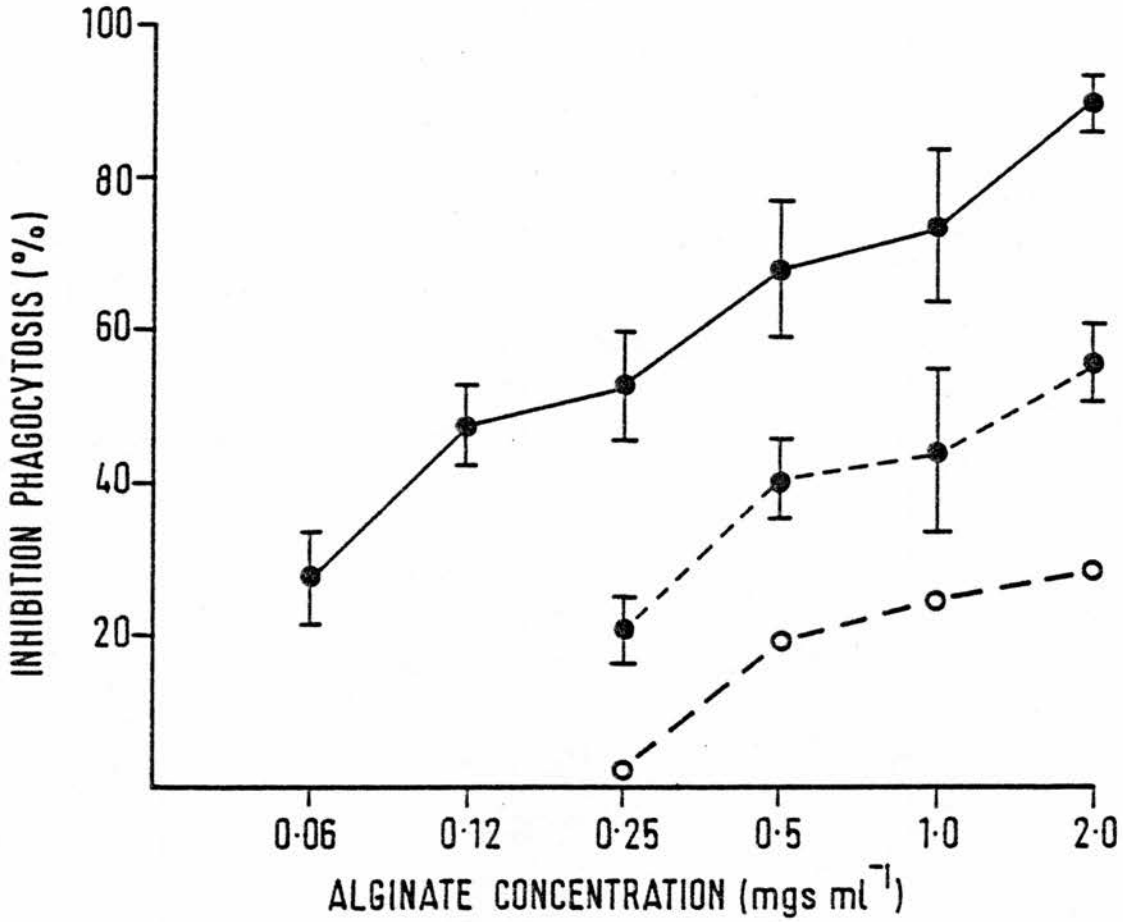


Figure 7

The effect of increasing concentrations of alginate on the phagocytosis of the non-mucoid P.aeruginosa 492a Rev1.

(●- -●) Pre-treatment of macrophage with alginate.

(●——●) Addition of alginate with bacteria to the monolayers.

Each point represents the mean ± 1 SEM of four experiments.

(O- -O) Alginate was vigorously washed from the macrophages prior to the addition of the bacteria. Each point represents the mean of two experiments.

4.0 THE EFFECT OF ALGINATE ON THE PHAGOCYTOSIS OF STAPH.ALBUS

The effect of alginate on the phagocytosis of Staph.albus was studied to investiage the specificity of the inhibiting action of the alginate. Staph.albus was opsonised at a concentration of 10^8 bacteria ml^{-1} with a 1/40 dilution of heat-inactivated pooled normal rat serum to give 30-40% phagocytosis. Pseudomonas alginate inhibited the phagocytosis ($P<0.05$) of Staph.albus when pre-incubated with the macrophage monolayer for 30 minutes (Figure 8). The degree of inhibition was related to the concentration of alginate and reached a maximum of 81% at 2mg ml^{-1}.

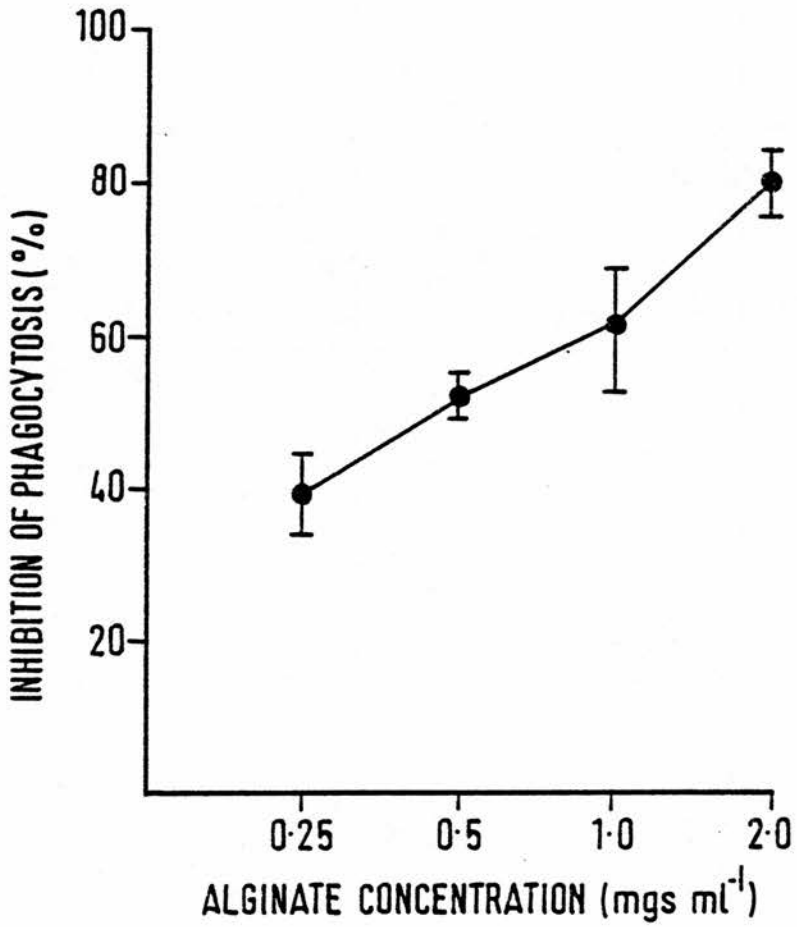


Figure 8

The effect of increasing concentrations of pseudomonas alginate on the phagocytosis of opsonised Staph.albus by rat alveolar macrophages. Each point represents the mean $\pm$ 1 SEM of four experiments.

5.0 THE EFFECT OF NON-PSEUDOMONAS ALGINATE ON THE PHAGOCYTOSIS OF P.AERUGINOSA

Alginic acid can be extracted from Macrocystis pyrifera (kelp) and shows structural similarities to pseudomonas alginate. The effect of this algal alginate on the phagocytosis of non-mucoid P.aeruginosa 492a Rev1 was investigated to determine if non-alginate products from P.aeruginosa 492a were the cause of the observed results with pseudomonas alginate.

Alginic acid inhibited the phagocytosis of the non-mucoid revertant in a dose dependent fashion when pre-incubated with the macrophage monolayer for 30 minutes (Figure 9). The observed inhibitory effect was reduced by extensive washing, in a manner similar to that observed for pseudomonas alginate (Figure 9). Concentrations of alginic acid above $2mgml^{-1}$ were used due to its low viscosity and inhibition reached 100% at $20mgml^{-1}$. Inhibition of phagocytosis also reached a maximum of 100% when the alginic acid was added to the macrophage monolayer together with the opsonised bacteria (Figure 10).

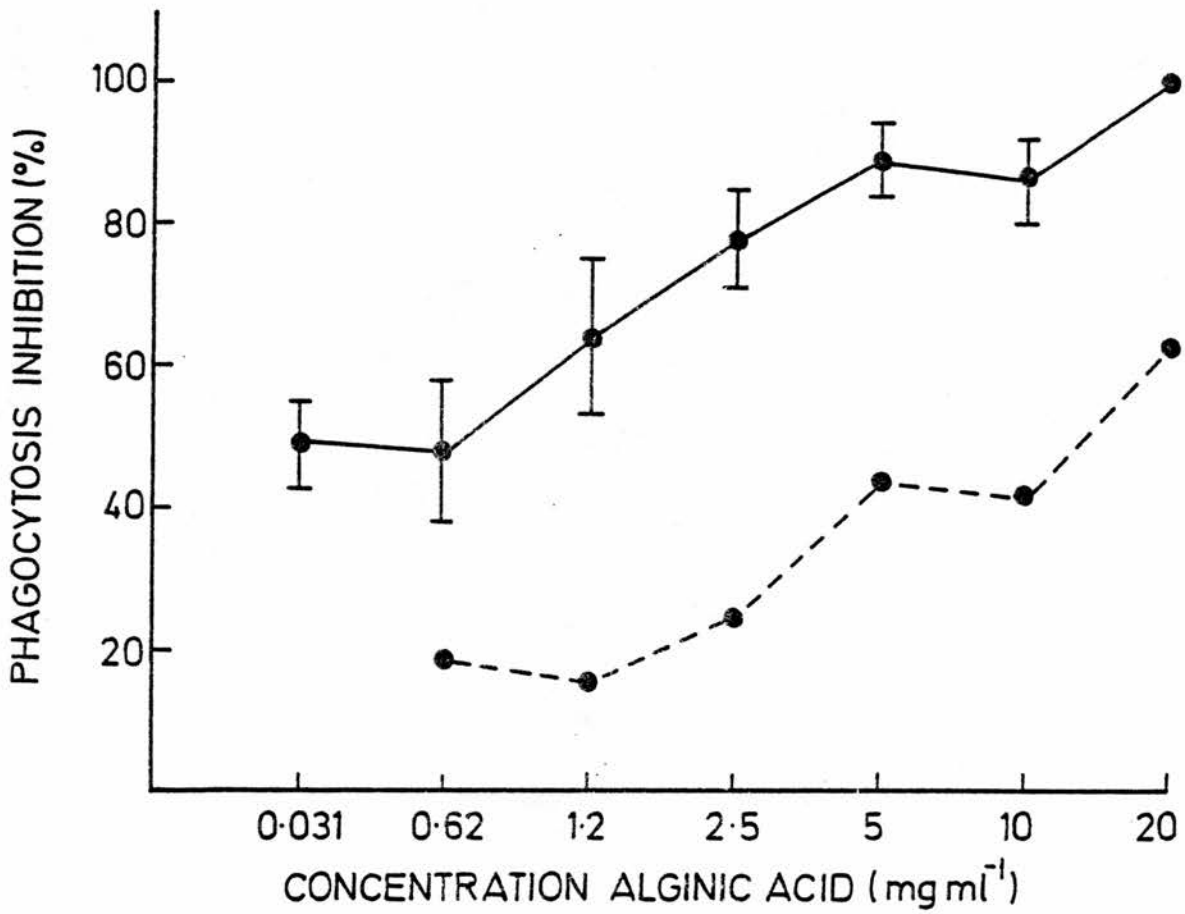


Figure 9

The effect of increasing concentrations of alginic acid on the phagocytosis of P.aeruginosa 492a Rev1. The macrophages were pre-incubated for 30 minutes with alginic acid and washed gently (●——●) or vigorously (●- -●). Each point represents the mean ± 1 SEM of three experiments (●——●) or the mean of two experiments (●- -●).

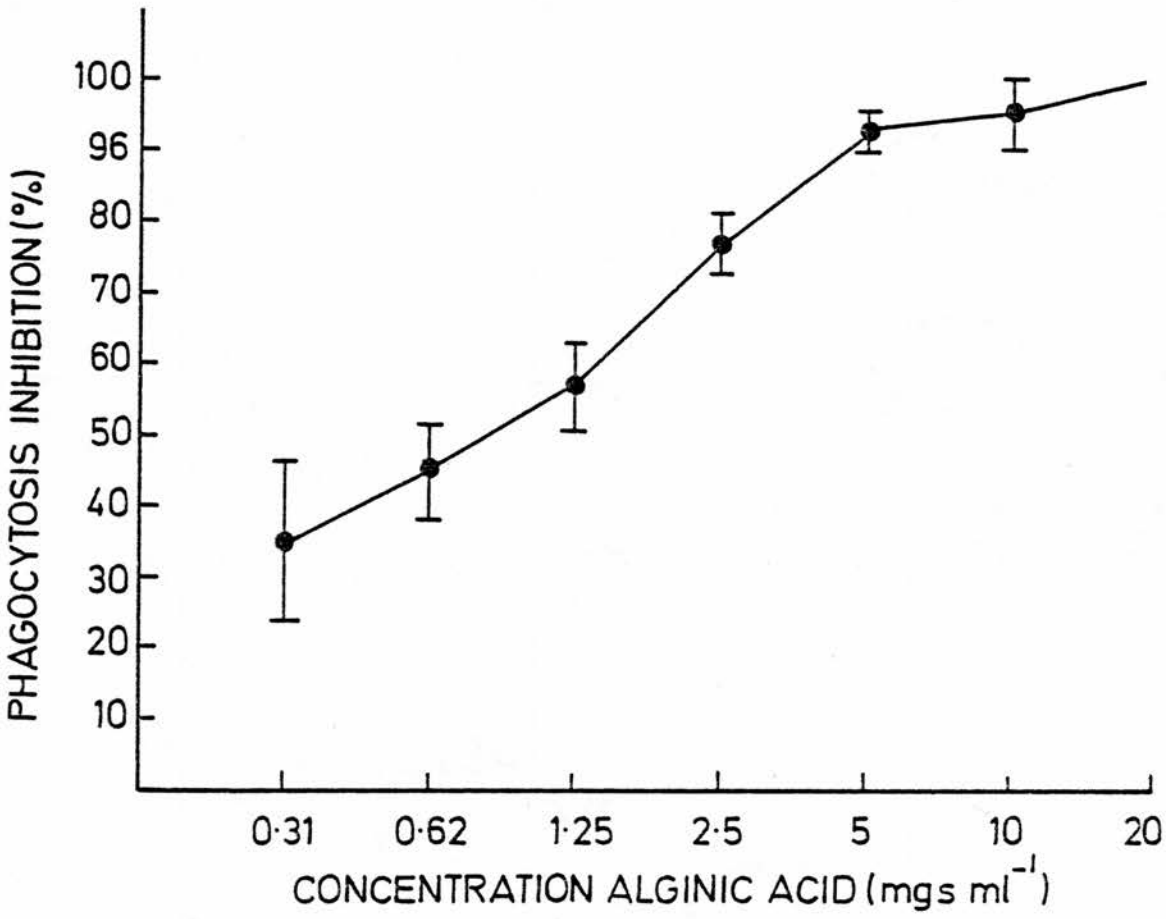


Figure 10

The effect of increasing concentrations of alginic acid on the phagocytosis of opsonised P.aeruginosa 492a Rev1, when added with the bacteria to the macrophage monolayers. Each point represents the mean ± 1 SEM of three experiments.

6.0 THE EFFECT OF ALGINATE ON CELL VIABILITY

The inhibitory effects of the alginate maybe a result of the toxicity of the substance to alveolar macrophages. Although this seemed unlikely as alginic acid had similar inhibitory effects on the phagocytosis of non-mucoid P.aeruginosa, the effect of pseudomonas alginate on cell viability was investigated.

At concentrations of $1mgml^{-1}$ and $2mgml^{-1}$ there was a slight, though not significant increase in cell death (Table 1) that may be a result of cell suffocation by the viscous alginate or cytolytic toxin contaminating the extract. However the percentage of cell death was not sufficient to account for the observed inhibitory effects on alveolar macrophage function.

Concentration alginate mg ml^{-1}	% Dead Cells
0	15 ± 4
0.25	15 ± 3 2
0.5	16 ± 5
1.0	20 ± 6
2.0	21 ± 5.2

TABLE I

The effect of alginate on cell viability. Alveolar macrophages were incubated for 30 minutes with alginate, washed and the viability estimated with trypan blue. Each figure represents the mean ± 1 SEM of three experiments.

7.0 SUMMARY

Non-mucoid P.aeruginosa 492a Rev1 required specific immune serum for opsonisation and subsequent phagocytosis by rat alveolar macrophages. Alginate extracted from a mucoid strain of P.aeruginosa inhibited the phagocytosis of the non-mucoid revertant and Staph. albus by the macrophages. Inhibition was observed when the cells were incubated with the alginate prior to addition of the bacteria and reduced if the alginate was removed from the cells by washing.

Alginic acid, an algal compound structurally similar to pseudomonas alginate inhibited phagocytosis in a similar manner, so that the observed inhibition is not likely to be a result of bacterial impurities in the pseudomonas alginate.

SECTION III

THE EFFECT OF ALGINATE ON BACTERIAL OPSONISATION

1.0 INTRODUCTION

As pseudomonas alginate was shown to inhibit phagocytosis it was considered possible that opsonisation might also be affected by binding of the alginate to the bacteria. Alginate has been shown to block the receptor for opsonic antibody on non-mucoid P.aeruginosa (Baltimore and Mitchell, 1980). However these workers found that the alginate had no effect on the opsonisation of non-mucoid strains when added to the bacteria and serum mixture.

2.0 THE EFFECT OF ALGINATE ON OPSONISATION

Alginate was added to the bacteria and serum mixture which was incubated for 20 minutes, washed and added to the macrophage monolayers. As unopsonised P.aeruginosa are not phagocytosed, any decrease in phagocytosis in this system would indicate an impairment of opsonisation.

Opsonisation of the non-mucoid revertant by immune serum was inhibited ($P < 0.05$) at 0.12 $mgml^{-1}$ and 0.25 $mgml^{-1}$ and almost totally inhibited ($P < 0.001$) at concentrations above 0.5 $mgml^{-1}$ (Figure 11). The inhibitory effect was not due to inadequate pelleting of the bacteria in the alginate and serum mixture during the washing procedure as colony counts showed that there was no difference in the control and experimental bacterial concentrations of the pellet.

Opsonisation of Staph.albus was inhibited by pseudomonas alginate but to a lesser extent than that observed with P.aeruginosa. This may be due to the fact that unopsonised Staph.albus are phagocytosed by alveolar macrophages. Therefore total inhibition of opsonisation will not result in a similar degree of inhibition of phagocytosis.

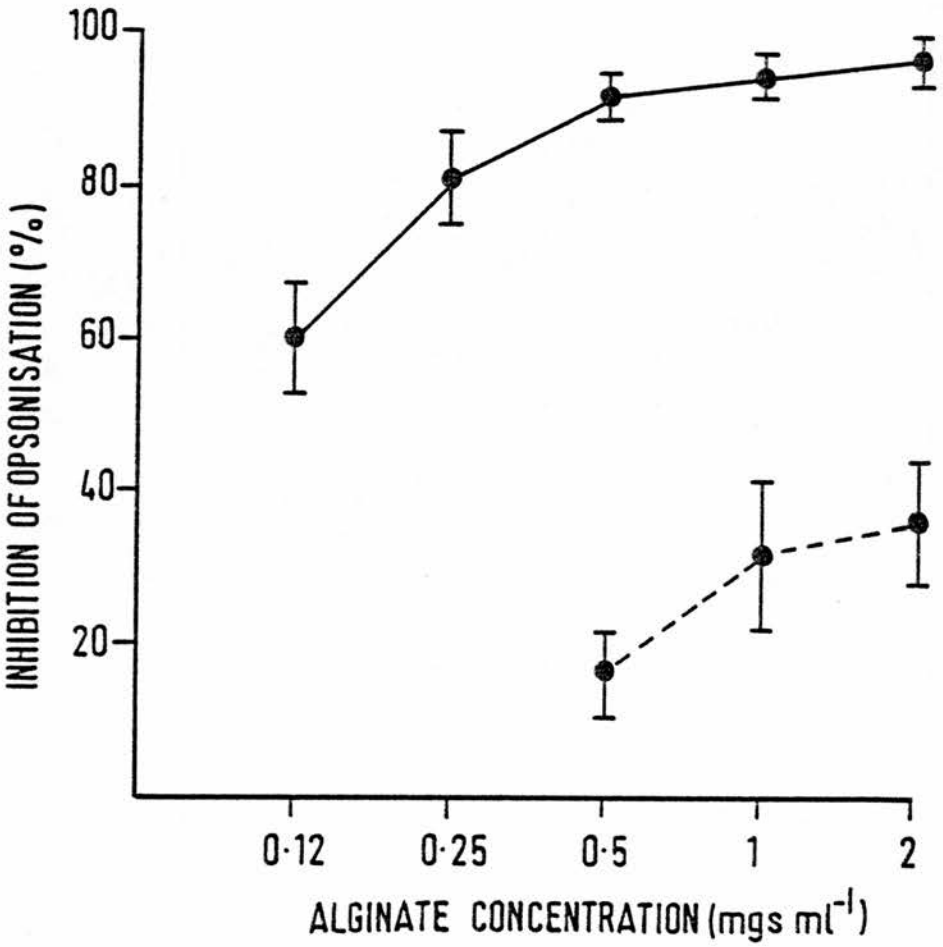


Figure 11

The effect of increasing concentrations of pseudomonas alginate on the opsonisation of non-mucoid P.aeruginosa (●——●) and Staph.albus (●- -●). Each point represents the mean ± 1 SEM of five experiments (P.aeruginosa) or three experiments (Staph.albus).

3.0 THE EFFECT OF CALCIUM CONCENTRATION ON INHIBITION OF OPSONISATION

Calcium ions cause pseudomonas alginate to gel and become more viscous in nature (Govan, 1983). Therefore the effect of calcium $^{++}$ concentration on the alginate-induced inhibition of opsonisation was investigated.

HBSS containing 2.5mm calcium^{++} (to correspond to the concentration present in the CF lung (Rabin, 1980)), 1.26mm calcium^{++} (normal HBSS concentration) and D.PBS containing no calcium^{++} was used during the opsonisation procedure.

There was no significant difference in the inhibition of opsonisation, measured by bacterial uptake into macrophages, at alginate concentrations of 0.5mgml^{-1} and 1 mgml^{-1}, between the three media concentrations of calcium^{++} (Figure 12).

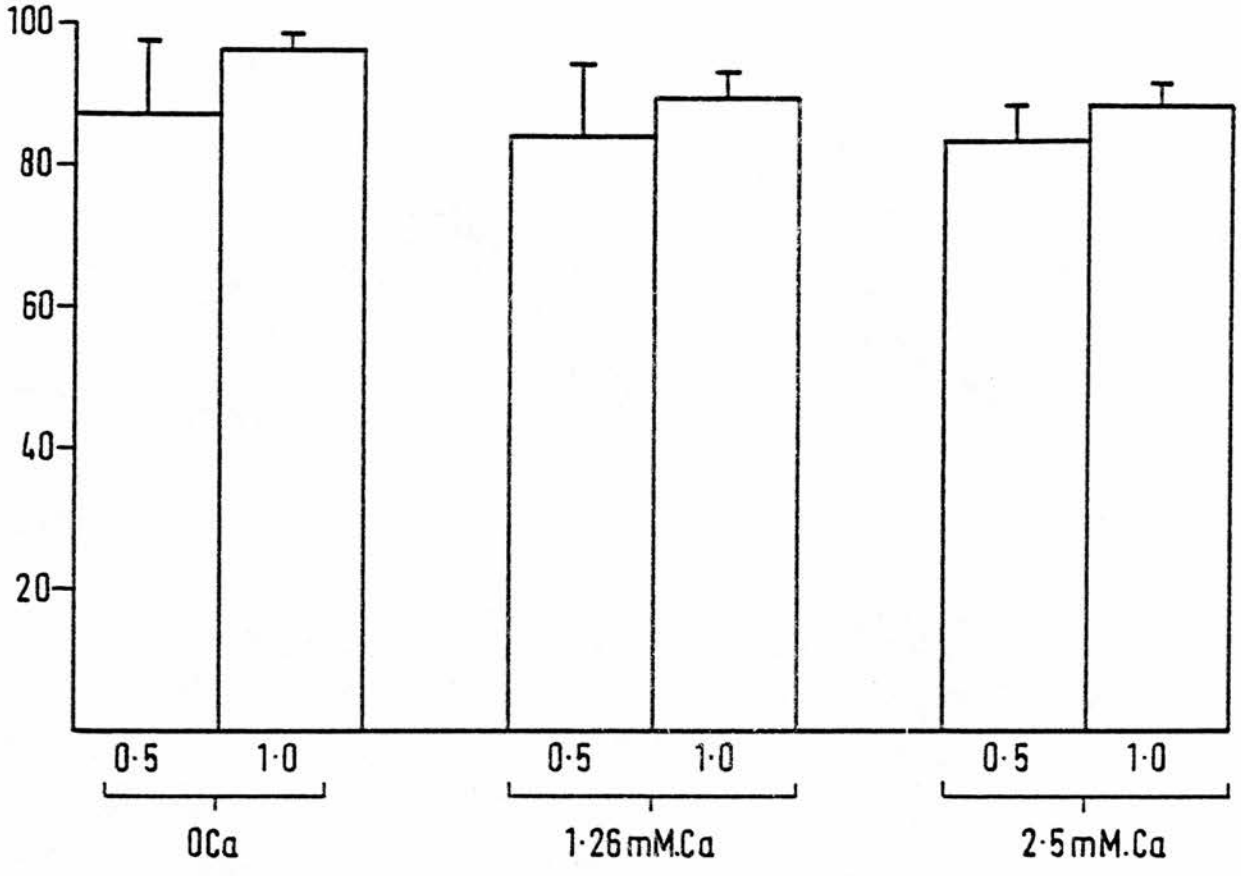


Figure 12

The effect of calcium on the inhibition of opsonisation caused by 0.5 or 1 mg ml^{-1} pseudomonas alginate. The y axis represents the percentage inhibition of opsonisation. Each column represents the mean $\pm$ 1 SEM of three experiments.

4.0 SUMMARY

Pseudomonas alginate inhibited the opsonisation of non-mucoid P.aeruginosa and Staph.albus in a dose-dependent fashion. Inhibition was not dependent on calcium^{++} concentration, as inhibition was still observed when calcium^{++} free medium was used throughout the opsonisation procedure.

SECTION IV

THE EFFECT OF ALGINATE ON THE BINDING OF ANTIBODY COATED SHEEP RED BLOOD CELLS TO Fc RECEPTORS AND THE PHAGOCYTOSIS OF LATEX PARTICLES

1.0 INTRODUCTION

In the previous sections, pseudomonas alginate was shown to inhibit the binding and phagocytosis of bacteria by rat alveolar macrophages and bacterial opsonisation. It was therefore postulated that the alginate was acting as a barrier, surrounding the macrophage and preventing the attachment of bacteria to the cell membrane. Investigation of this hypothesis was carried out by studying the effect of alginate on binding of antibody coated sheep red blood cells (EA_G) to Fc receptors and the nonspecific uptake of latex particles by alveolar macrophages.

2.0 DETERMINATION OF THE OPTIMAL CONDITIONS FOR THE DETECTION OF Fc RECEPTORS

Antibody-coated sheep red blood cells(EA_G) were used to detect the presence of Fc receptors. Macrophages were incubated with serum-free Eagle's MEM. This was essential as the presence of foetal calf serum (10%) in the medium enhanced ($P < 0.05$) the expression of Fc receptors on rat alveolar macrophages (Figure 13). The antibody used in this experiment was an IgG against sheep red blood cells, raised in rabbits (donated by G.Dougherty, Bacteriology Department). The observed increase in Fc receptors may be a result of the rounding up of the macrophages caused by the foetal calf serum. The Fc receptors would then be closer together and more likely to bind sensitised erythrocytes. Foetal calf serum had no effect on Fc receptor expression of human peripheral blood monocytes (results not shown), which are smaller and more rounded than alveolar macrophages.

The antibody used in all other experiments was a monoclonal IgG2b raised in mice against sheep erythrocytes (sera-lab). Binding of sensitised erythrocytes was directly related to the concentration of antibody used and reached a maximum of 62.5% at a 1/40 dilution (Figure 14). Human peripheral blood monocytes had lower avidity Fc receptors than rat alveolar macrophages, although a maximum of 57.5% binding was reached at an antibody dilution of 1/40.

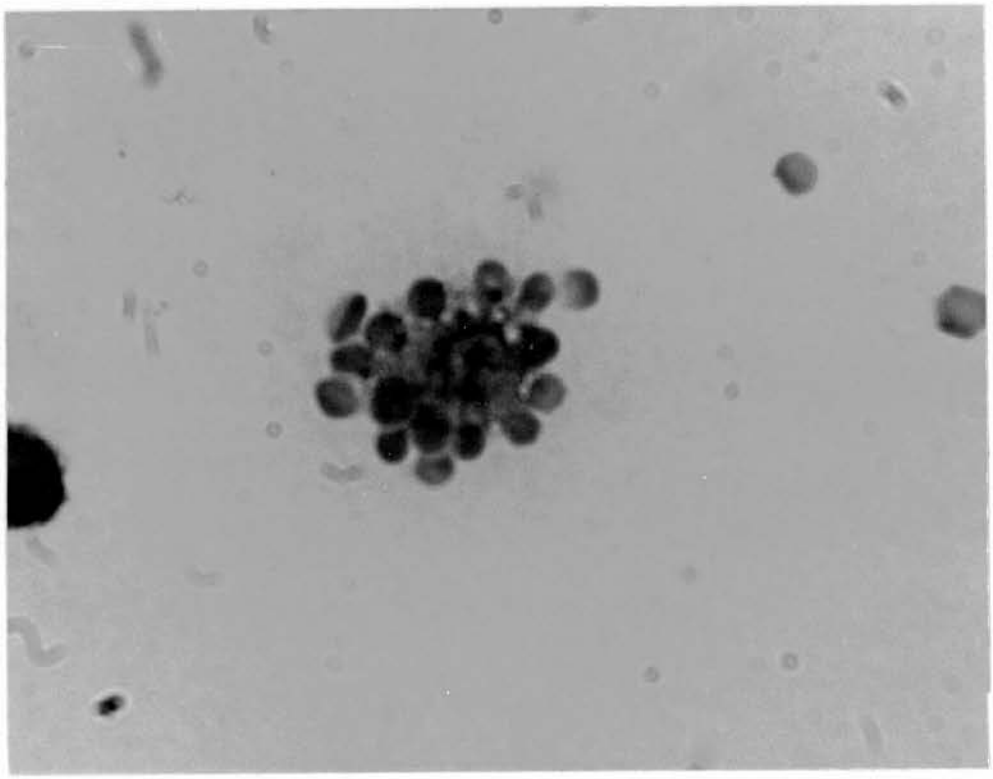


PLATE IV

Rat alveolar macrophage rosette formation with EA_G (prepared with mouse IgG2b monoclonal as the sensitising antibody). (May Grunwald/Giemsa, x 1,250 magnification.)

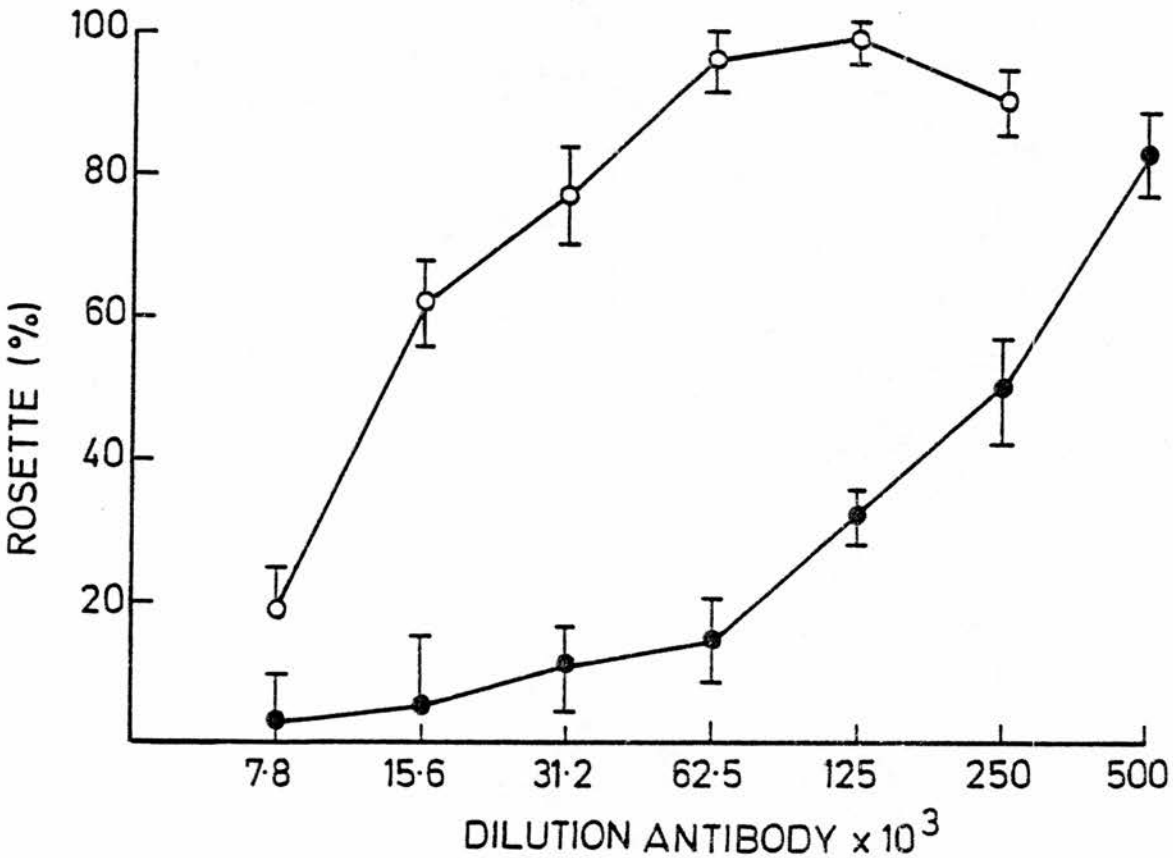


Figure 13

The effect of increasing concentrations of IgG antibody on rosette formation by alveolar macrophages incubated in Eagles MEM without serum (●——●) or containing 10% foetal calf serum (O——O). Each point represents the mean ± 1 SEM of three experiments.

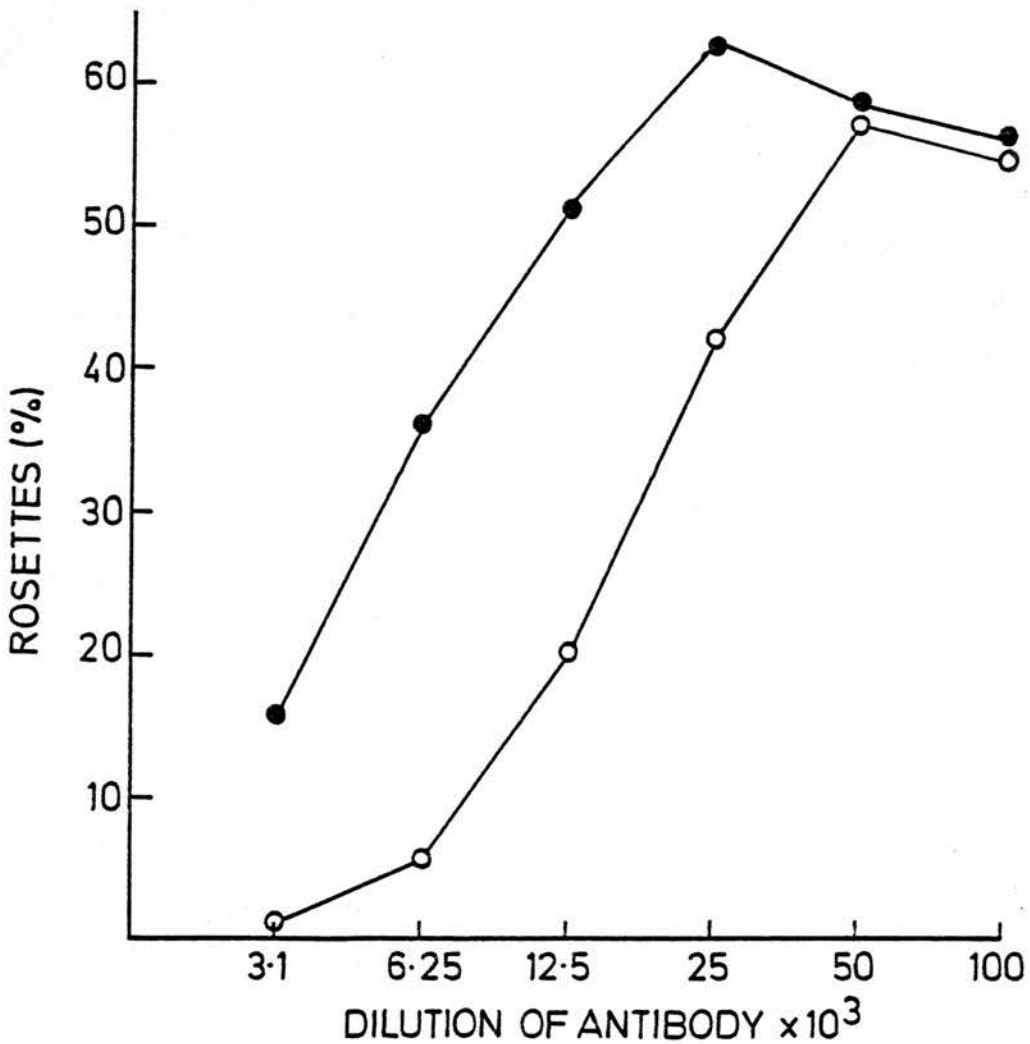


Figure 14

The effect of increasing the amount of sensitising antibody (IgG2b) on the binding of EA_G by rat alveolar macrophages (●——●) and human peripheral blood monocytes (○——○). Each point represents the mean of two experiments.

3.0 THE EFFECT OF ALGINATE ON Fc RECEPTORS

The effect of pseudomonas alginate on Fc receptors was studied with an antibody dilution that gave 30-40% binding of EA_G to the alveolar macrophages.

Alginate reduced ($P < 0.05$) the binding of EA_G to the macrophages (Figure 15). This inhibition was greater when the alginate was incubated together with the sheep erythrocytes than when the macrophages were pre-treated for 30 minutes.

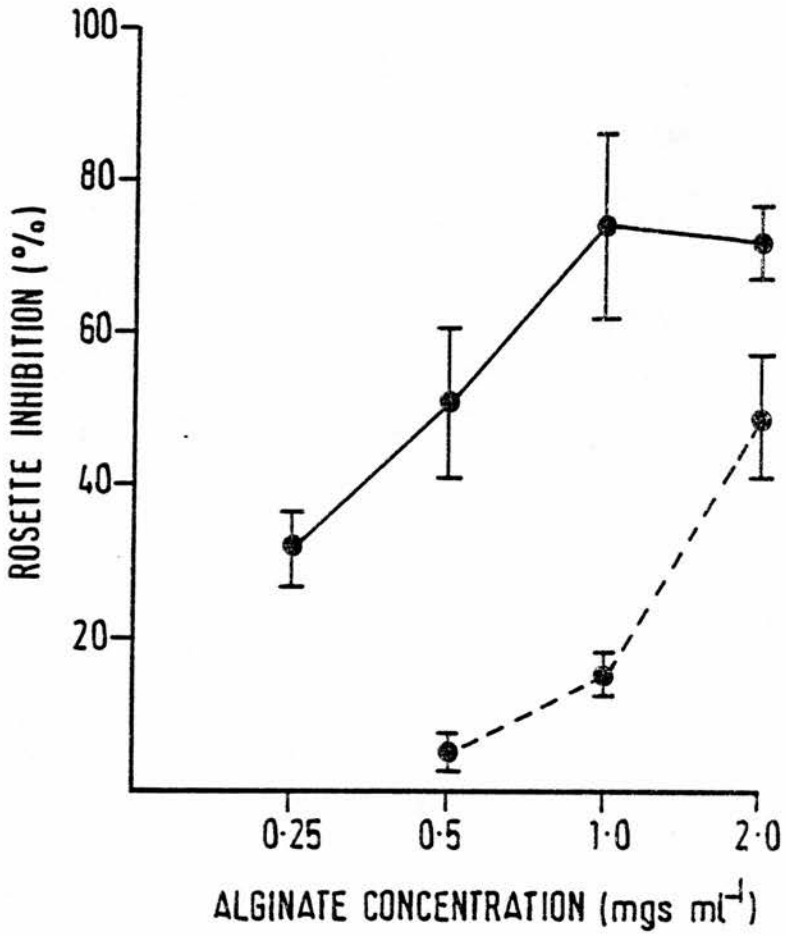


Figure 15

The effect of increasing concentrations of pseudomonas alginate on rosette formation by EA_G to alveolar macrophages.

(●– – –●) Pretreatment of macrophages with alginate.

(●————●) Addition of alginate with EA_G to the monolayers. Each point represents the mean $\pm$ 1 SEM of four experiments.

4.0 THE EFFECT OF ALGINATE ON THE PHAGOCYTOSIS OF LATEX PARTICLES

Pseudomonas alginate inhibited the phagocytosis of latex particles by rat alveolar macrophages in a dose-dependent fashion (Figure 16). When the macrophages were pre-incubated for 30 minutes, phagocytosis was inhibited ($P < 0.05$) at $1mgml^{-1}$ and $2mgml^{-1}$. Inhibition was increased at all concentrations used when the alginate was added with the latex to the macrophages, ($P < 0.05$) at $0.25mgml^{-1}$ and $0.5mgml^{-1}$ and ($P < 0.01$) at $1mgml^{-1}$ and $2mgml^{-1}$.

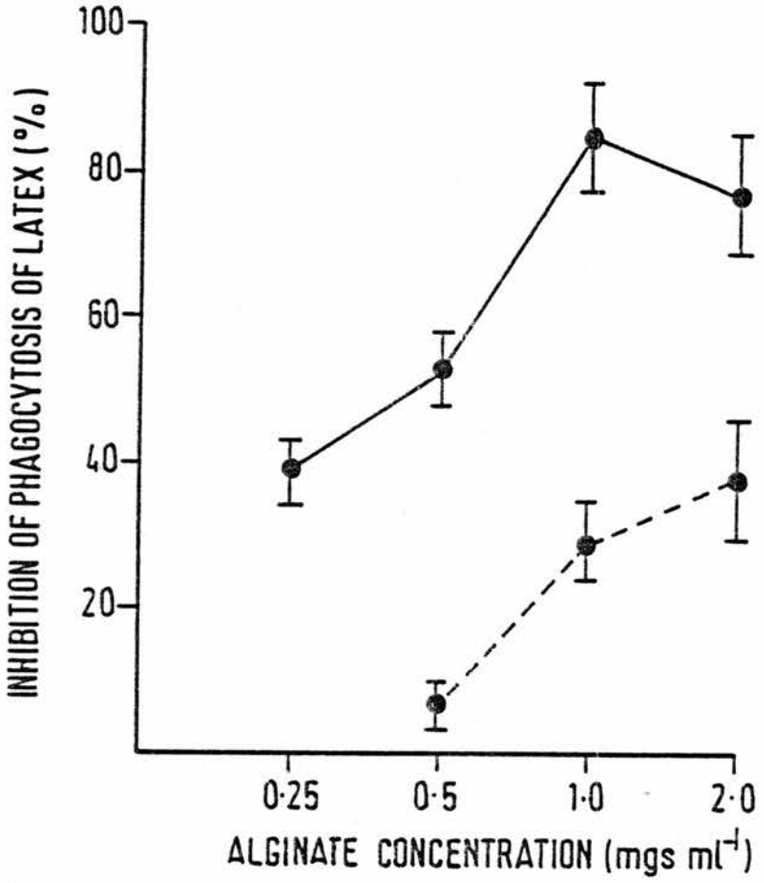


Figure 16

The effect of increasing concentrations of alginate on the phagocytosis of latex particles by pseudomonas alginate.

(●---●) Pre-treatment of macrophages with alginate.

(●——●) Addition of alginate with latex particles to the monolayers

Each point represents the mean ± 1 SEM of three experiments.

5.0 SUMMARY

Pseudomonas alginate inhibited the binding of sensitised sheep erythrocytes to alveolar macrophage Fc receptors and the phagocytosis of latex particles by these cells. These results support the hypothesis that the alginate binds nonspecifically to the macrophage and prevents particle attachment. Greater inhibition was observed when the alginate was added along with the indicator cells. The test particles may be trapped by the viscous mucoid material and prevented from coming into contact with the macrophages.

PART B

THE EFFECT OF ANTIBIOTICS ON RAT ALVEOLAR MACROPHAGES AND HUMAN PERIPHERAL BLOOD MONOCYTES

1.0 INTRODUCTION

In Part A it was shown that alginate extracted from a mucoid strain of P.aeruginosa had an inhibitory effect on the phagocytosis and opsonisation of bacteria. If these effects occur in vivo, they may partly explain the predominance of mucoid P.aeruginosa in CF patients. However the results do not take into account the effect of antibiotic therapy on the bacteria or the host. Although antibiotic treatment may improve the clinical condition of the CF patient, the bacteria are rarely exadicated from the lung and re-infection occurs (Kulezycki et al., 1978). One of the reasons for this may be that antibiotics penetrate poorly through the thick bronchial secretions that occur in CF patients, so that levels above the MIC are rarely achieved in the CF lung. Levels of antibiotics obtained in CF sputum are low compared to those found in serum and often below the MIC (Malmborg et al., 1981).

Conventional antimicrobial sensitivity testing only considers the interaction between drug and micro-organisms, generally ignoring possible effects that the antibiotic may have on the host immune defense mechanisms. Tobramycin, azlocillin and ticarcillin are three antibiotics that have been widely used during CF therapy for chronic P.aeruginosa lung infection. The purpose of this study was to investigate the possibility that the low levels of these antibiotics achieved in the CF lung may affect alveolar macrophage function. As shown in Part A, macrophage function may already be impaired by alginate produced by mucoid P.aeruginosa that are infecting the CF lung.

SECTION I

THE EFFECT OF TOBRAMYCIN, AZLOCILLIN AND TICARCILLIN ON THE PHAGOCYTOSIS OF P.AERUGINOSA BY RAT ALVEOLAR MACROPHAGES.

1.0 INTRODUCTION

The MIC of each antibiotic was determined as described in Materials and Methods, the values being given in Table II. Concentrations of the three antibiotics above and below the MIC were studied in the range 1/8 MIC - 8 x MIC value. These concentrations are well within the therapeutic range and similar to the concentration of antibiotics obtained in the lungs of CF patients (Malmborg et al., 1981; Hoogkamp-Korstanje and van der Laag, 1983). The antibiotics were either pre-incubated with the alveolar macrophages or added to the monolayers with the opsonised bacteria.

Antibiotic	MIC $\mu gm\ ml^{-1}$
Tobramycin	0.3
Azlocillin	5.0
Ticarcillin	20.0

Table II

Minimum inhibitory concentrations (MIC) of the antibiotics used in this study. The concentration is that which inhibited growth of an overnight culture of P.aeruginosa 492 a Rev 1. Each antibiotic was tested three times and at regular intervals.

2.0 THE EFFECT OF THE ANTIBIOTICS ON PHAGOCYTOSIS-DOSE RESPONSES

The optimal conditions for phagocytosis of P.aeruginosa have already been described in Part A. Azlocillin and tobramycin inhibited the phagocytosis of P.aeruginosa 492a Rev1 when they were added to the alveolar macrophage monolayers at the same time as the opsonised bacteria. This inhibition was significant ($P < 0.05$) at 1/4MIC to 8 x MIC values for azlocillin (Figure 17) and at 1/8MIC to 1/2MIC values for tobramycin (Figure 18).

To determine whether the antibiotics were having a direct effect on the phagocytes, macrophage monolayers were incubated with tobramycin, azlocillin and ticarcillin for 30 minutes prior to the addition of bacteria. Azlocillin enhanced phagocytosis at 1/4MIC ($P < 0.05$) and MIC ($P < 0.01$) values but at other concentrations studied had no effect (Table III).

Figure 19 shows that the phagocytosis of opsonised P.aeruginosa was inhibited by tobramycin. This effect was significant at 1/8MIC and MIC values ($P < 0.05$) and at 1/4MIC, 1/2MIC and 2 x MIC values ($P < 0.01$). Inhibition of phagocytosis was still observed after extensive washing of the monolayers to ensure the removal of any extracellular antibiotic. However, the values returned to control levels when the macrophages were re-incubated in normal Eagles MEM without antibiotics (Table IV).

Ticarcillin had no effect on the phagocytosis of P.aeruginosa with either treatment at the concentrations studied (Table V).

The observed effects were not a result of any toxicity as neither tobramycin or azlocillin affected cell viability as measured by trypan blue exclusion.

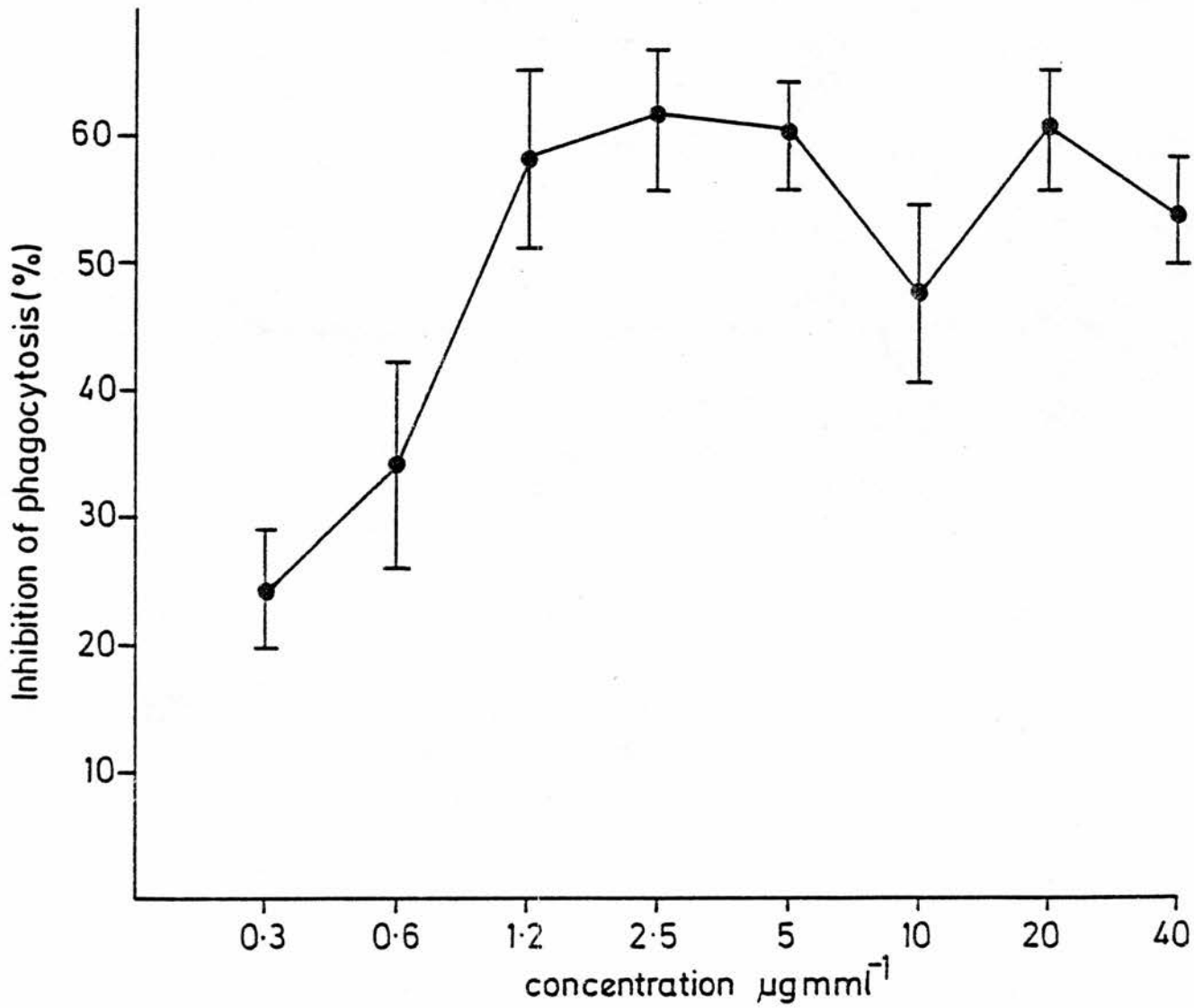


Figure 17

The effect of increasing concentrations of azlocillin on the phagocytosis of P.aeruginosa by rat alveolar macrophages, when the antibiotic was added with the bacteria to the monolayers. Each point represents the mean ± 1SEM of three experiments.

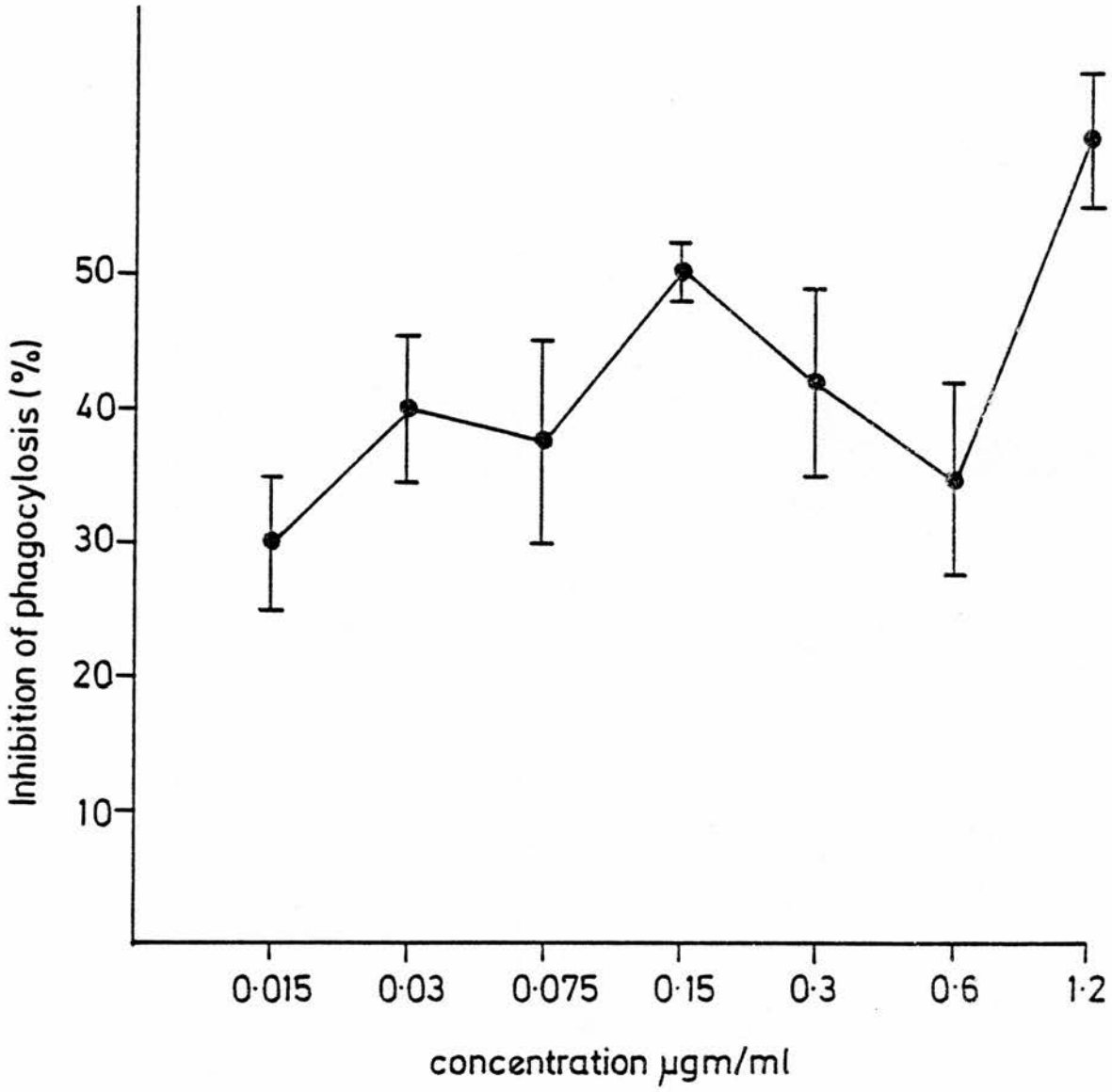


Figure 18

The effect of increasing concentrations of tobramycin on the phagocytosis of P.aeruginosa by rat alveolar macrophages when added with the bacteria to the monolayers. Each point represents the mean ± 1 SEM of three experiments.

Concentration $\mu gm\ ml^{-1}$	Phagocytosis % Enhancement	Binding EA_G % Enhancement
0.6	48.5 ± 13	8.3 ± 10.5
1.2	56.5 ± 13.7	16.7 ± 7.8
2.5	39.1 ± 7.8	12.1 ± 8
5.0	69.8 ± 15.6*	20.7 ± 10.7
10.0	9.8 ± 2.7	9.4 ± 7.6
20.0	28.5 ± 8.3	6.5 ± 8.3
40.0	40.6 ± 14	5.4 ± 7.5

*$p < 0.05$

TABLE III

The effect of azlocillin on alveolar macrophages. Macrophage monolayers incubated with azlocillin for 30 minutes prior to assays. Each figure represents the mean of three experiments (phagocytosis) or four experiments. (Binding EA_G) ± 1 SEM.

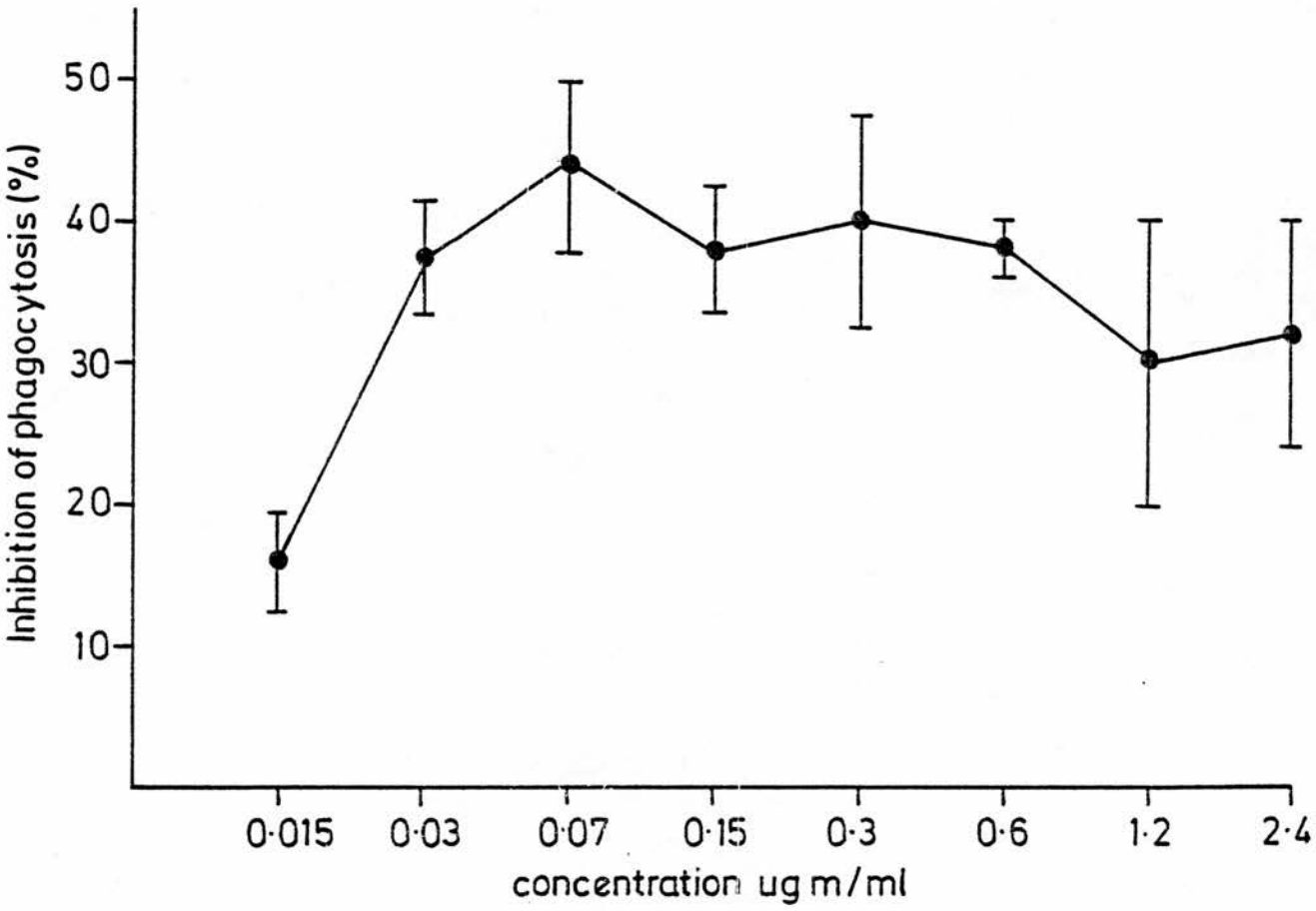


Figure 19

The effect of increasing concentrations of tobramycin on phagocytosis by rat alveolar macrophages. The macrophages were incubated with antibiotic for 30 minutes, and washed before addition of P.aeruginosa. Each point represents the mean $\pm$ 1 SEM of four experiments.

Treatment		Phagocytosis % Inhibition
Wash well		27.4 ± 6.2
Re-incubate macrophages after washing	10 minutes	30.0 ± 4.8
	20 minutes	10.0 ± 2.4
	30 minutes	0.9 ± 2.2

TABLE IV

Effect of washing and re-incubation on tobramycin treatment of macrophages. Monolayers were treated for 30 minutes with 0.15 μgm ml^{-1} tobramycin. Some monolayers were vigorously washed (five times) and bacteria added. Other monolayers were washed twice and incubated in fresh Eagles MEM for various times prior to addition of bacteria. Each figure represents the mean of three experiments ± 1 SEM.

Concentration $\mu gm\ ml^{-1}$	Added with bacteria to macrophages	Pre-treatment of macrophages
2.5	24.9 ± 7.0	4.8 ± 6.6
5	24.7 ± 11.1	12.8 ± 10.0
10	7.6 ± 10.2	26.8 ± 11.9
20	10.6 ± 10.5	6.1 ± 10.2
40	10.0 ± 13.3	2.6 ± 7.8
80	14.4 ± 12.5	11.3 ± 11.1
160	19.0 ± 12.4	13.0 ± 15.2

TABLE V

The effect of ticarcillin on the phagocytosis of opsonised P.aeruginosa by rat alveolar macrophages. Each figure shows the % inhibition and represents the mean ± 1 SEM of three experiments.

3.0 THE EFFECT OF TOBRAMYCIN ON "LECTIN-LIKE" RECEPTORS

When tobramycin was incubated for 30 minutes with rat alveolar macrophages, prior to the addition of unopsonised P.aeruginosa, the binding of the bacteria was inhibited ($P < 0.05$) (Figure 20). This inhibition was maximal at 2 x MIC, at which value, the level of inhibition reached 65% ($P < 0.01$). Therefore tobramycin has an effect on the binding of unopsonised bacteria similar to that observed for the phagocytosis of opsonised bacteria. However the effect of tobramycin on the "lectin-like" receptor was not pursued as phagocytosis of P.aeruginosa does not occur via this receptor on rat alveolar macrophages.

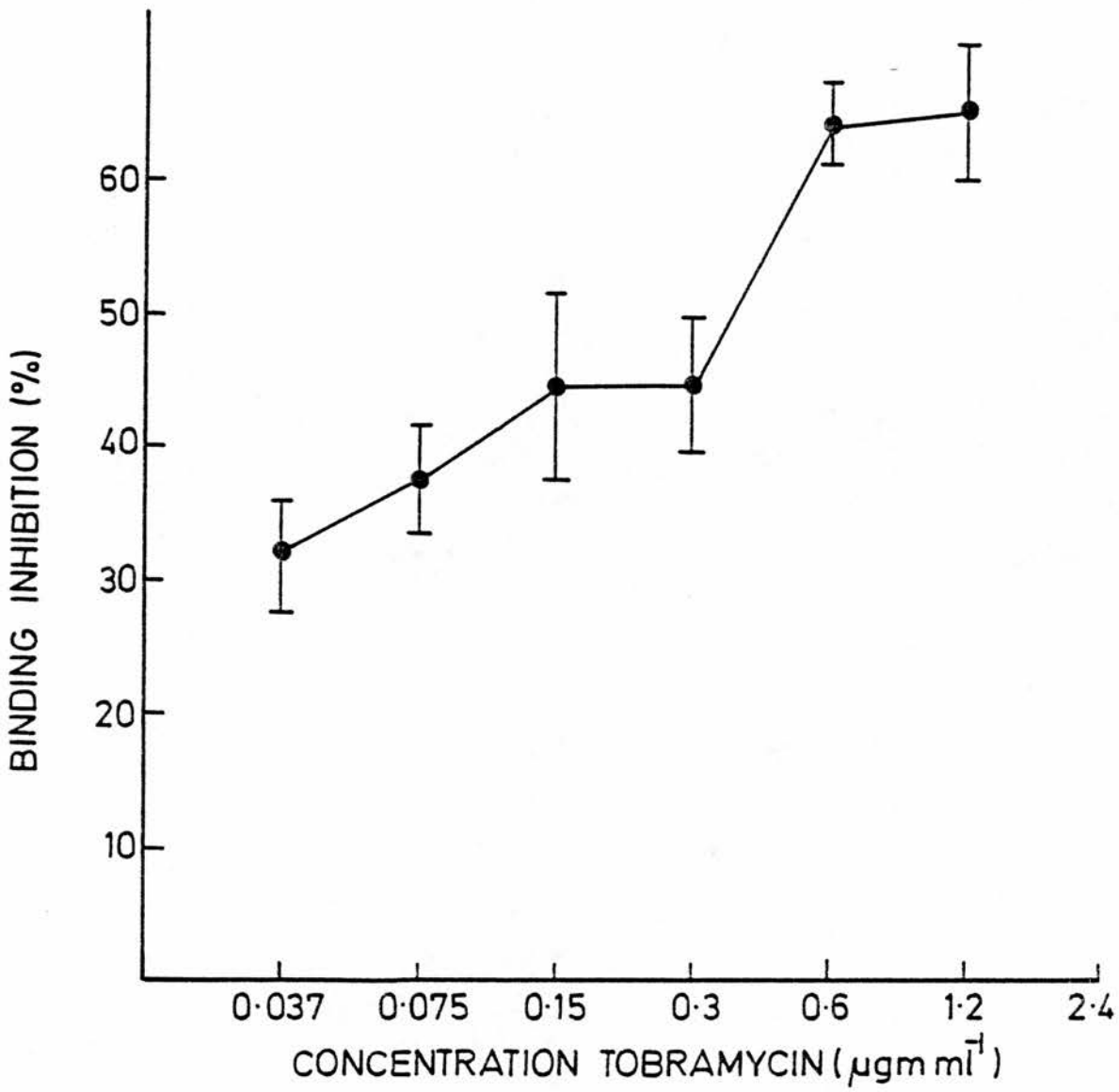


Figure 20

The effect of increasing concentrations of tobramycin on binding of unopsonised P.aeruginosa to rat alveolar macrophages. The macrophages were incubated for 30 minutes with antibiotic prior to addition of the bacteria. Each point represents the mean ± 1 SEM of three experiments.

4.0 TIME COURSE OF THE INHIBITION OF THE PHAGOCYTOSIS AND BACTERIAL BINDING BY TOBRAMYCIN.

The effect of 1/2MIC value (0.15 μgm ml^{-1}) of tobramycin on alveolar macrophages was studied when the antibiotic was incubated with the cells at various times. Phagocytosis was inhibited when the macrophages were incubated for 15 minutes to 120 minutes. However this inhibition was only significant ($P < 0.05$) up to 60 minutes. By 120 minutes inhibition was reduced and the level of phagocytosis had almost returned to the original value (Figure 21). Inhibition of the binding of unopsonised P.aeruginosa occurred at the four time intervals ($P < 0.05$) with the maximal response occurring at 60 minutes ($P < 0.01$).

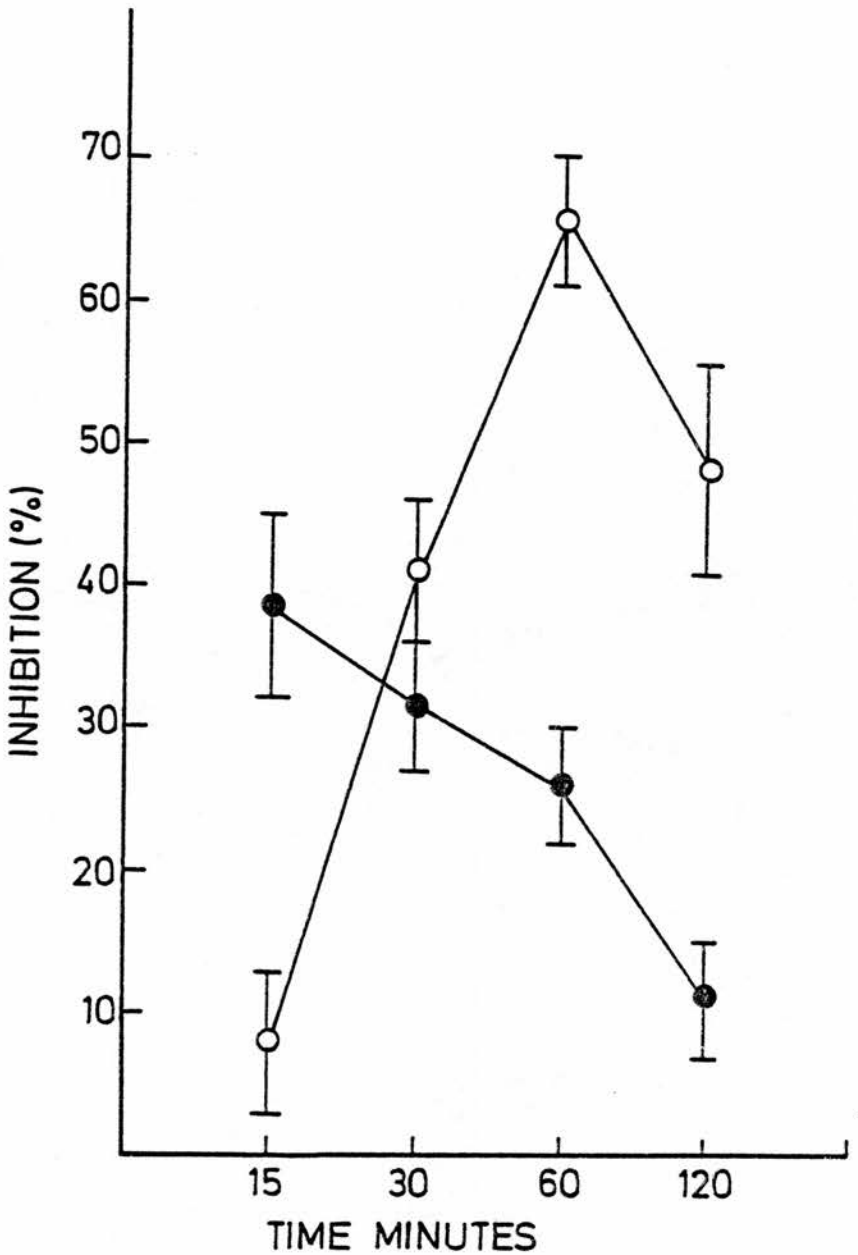


Figure 21

The time course of the percentage inhibition of phagocytosis of opsonised bacteria (●——●) and binding of unopsonised bacteria (O——O) by alveolar macrophages, after pre-treatment with 1/2MIC value of tobramycin. Each point represents the mean ± 1 SEM of three experiments.

5.0 SUMMARY

Tobramycin inhibited the phagocytosis of opsonised P.aeruginosa and the binding of unopsonised P.aeruginosa when pre-incubated with macrophage monolayers for 30 minutes. These effects were dose and time-dependent. Azlocillin and ticarcillin had no effect on the phagocytosis of P.aeruginosa when the antibiotics were pre-incubated with the macrophage monolayers.

Inhibition of phagocytosis of P.aeruginosa was observed when tobramycin or azlocillin was co-incubated with the macrophage monolayers and opsonised bacteria while ticarcillin had no effect.

SECTION II

THE EFFECT OF TOBRAMYCIN, AZLOCILLIN AND TICARCILLIN ON THE BINDING OF EA_G TO RAT ALVEOLAR MACROPHAGE Fc RECEPTORS

1.0 INTRODUCTION

As tobramycin and azlocillin had an effect on the phagocytic function of macrophages, which in this case was mediated by the Fc receptor, the effect of the antibiotics on the receptor was investigated. To ensure specificity the sheep erythrocytes were sensitised with a mouse monoclonal IgG2b. Optimal conditions for the binding of EA_G to macrophages were described in Part A. The antibiotics were either pre-incubated with the macrophage monolayers or added to the monolayers at the same time as the erythrocytes.

2.0 THE EFFECT OF ANTIBIOTICS ON FcRECEPTORS - DOSE RESPONSE

Azlocillin and tobramycin both inhibited the binding of EA_G to macrophage monolayers when added with the sheep erythrocytes. With azlocillin the effect was significant ($P < 0.05$) until a maximal inhibition occurred at 1/4MIC, thereafter declining until at 4 x MIC value, the effect was not significant (Figure 22).

For tobramycin the effect was dose-dependent reaching a maximum at the MIC value. Inhibition was significant ($P < 0.01$) at 1/4MIC, to 2 x MIC values (Figure 23).

Pre-treatment of the macrophages for 30 minutes with tobramycin resulted in enhanced binding of EA_G, which was significant ($P < 0.05$) at 1/8MIC to 2 x MIC (Figure 24). Azlocillin had no effect on the binding of EA_G to the macrophages when it was pre-incubated with the cells for 30 minutes (Table III).

As with phagocytosis, ticarcillin had no effect on the binding of EA_G with either treatment (Table VI).

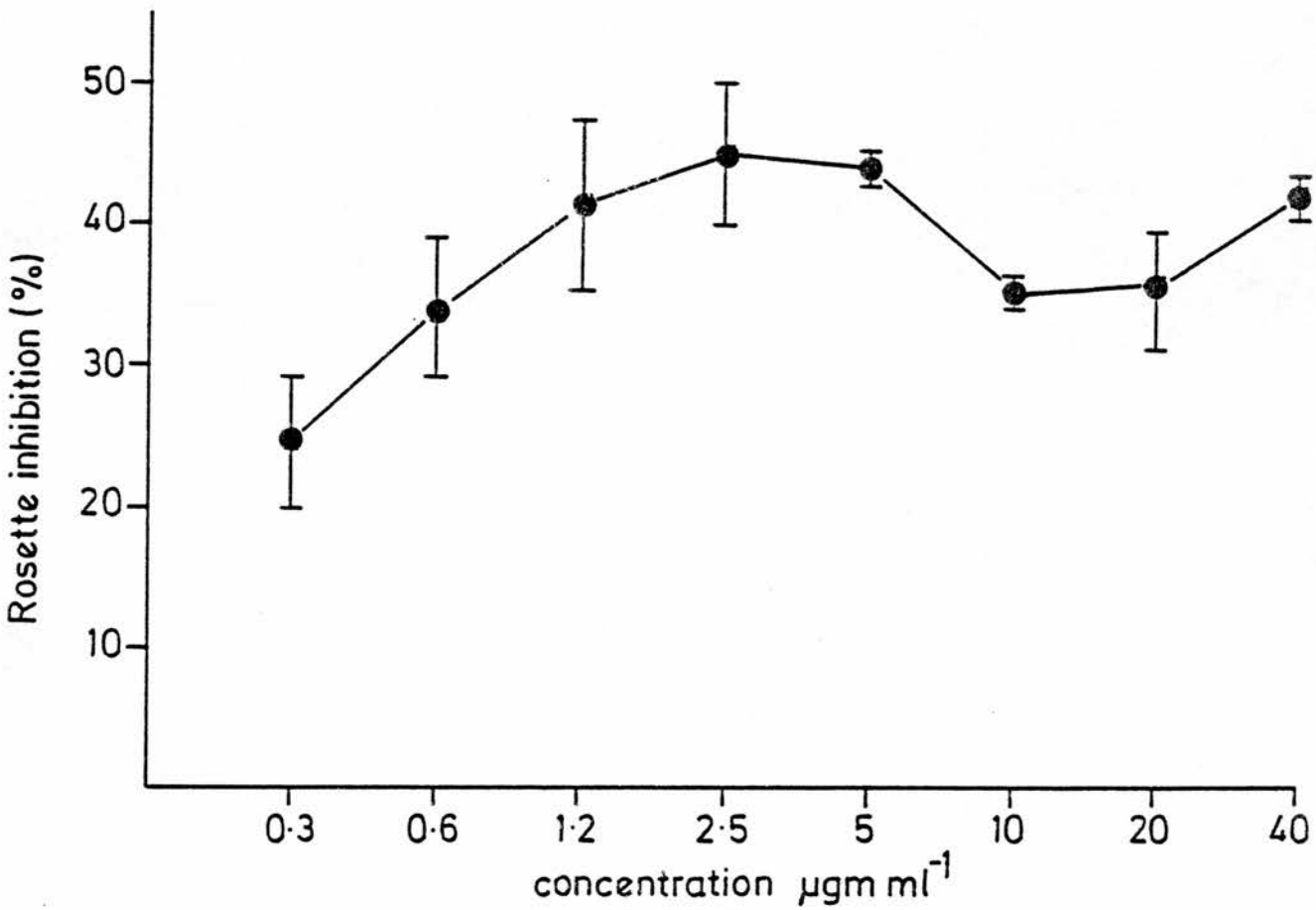


Figure 22

The effect of increasing concentrations of azlocillin on the binding of EA_G to rat alveolar macrophages, when the antibiotic was added with the erythrocytes. Each point represents the mean $\pm$ 1 SEM of four experiments.

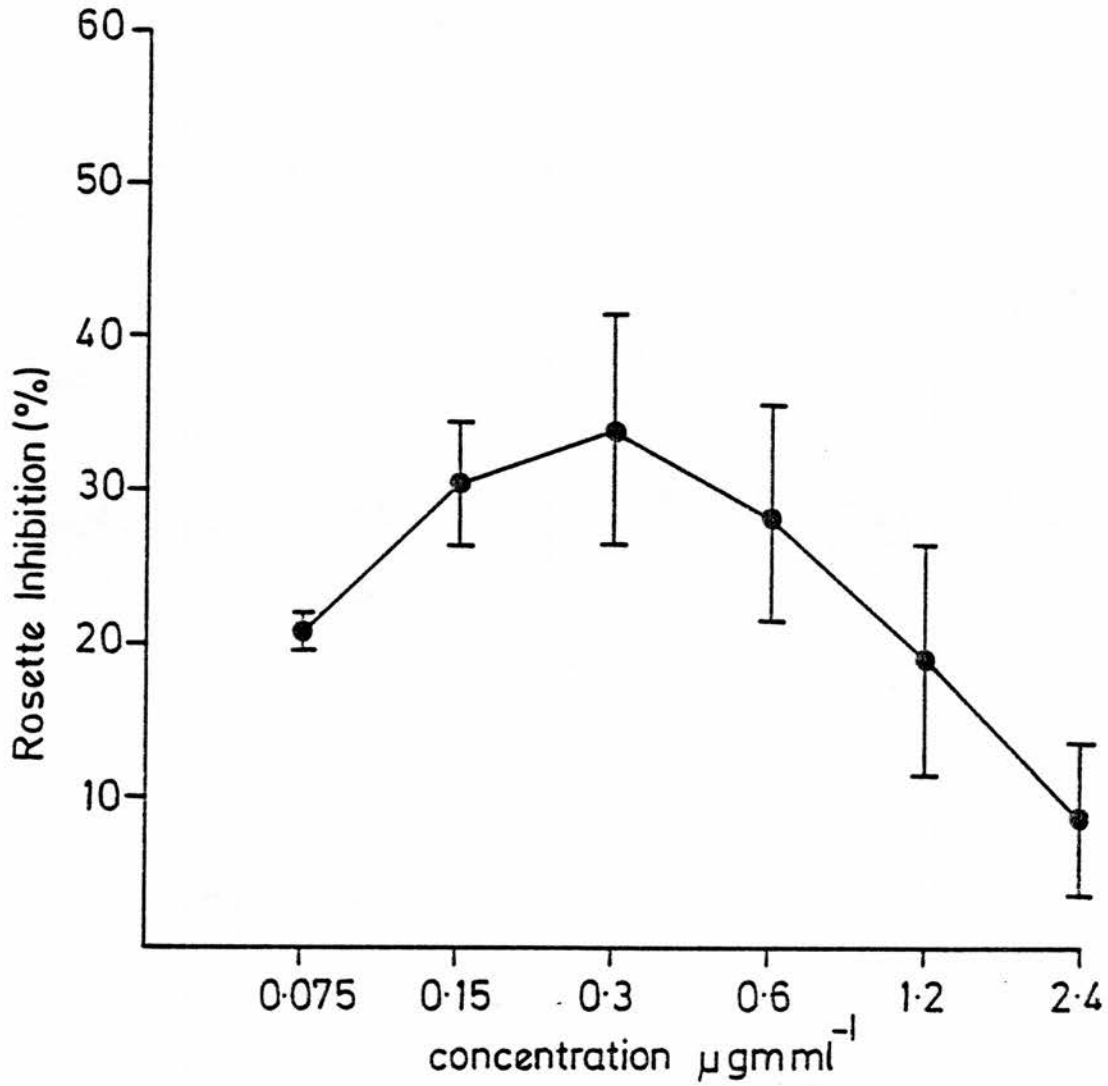


Figure 23

The effect of increasing concentrations of tobramycin on the binding of EA_G to rat alveolar macrophages when the antibiotic was added with the erythrocytes. Each point represents the mean $\pm$ 1 SEM of three experiments.

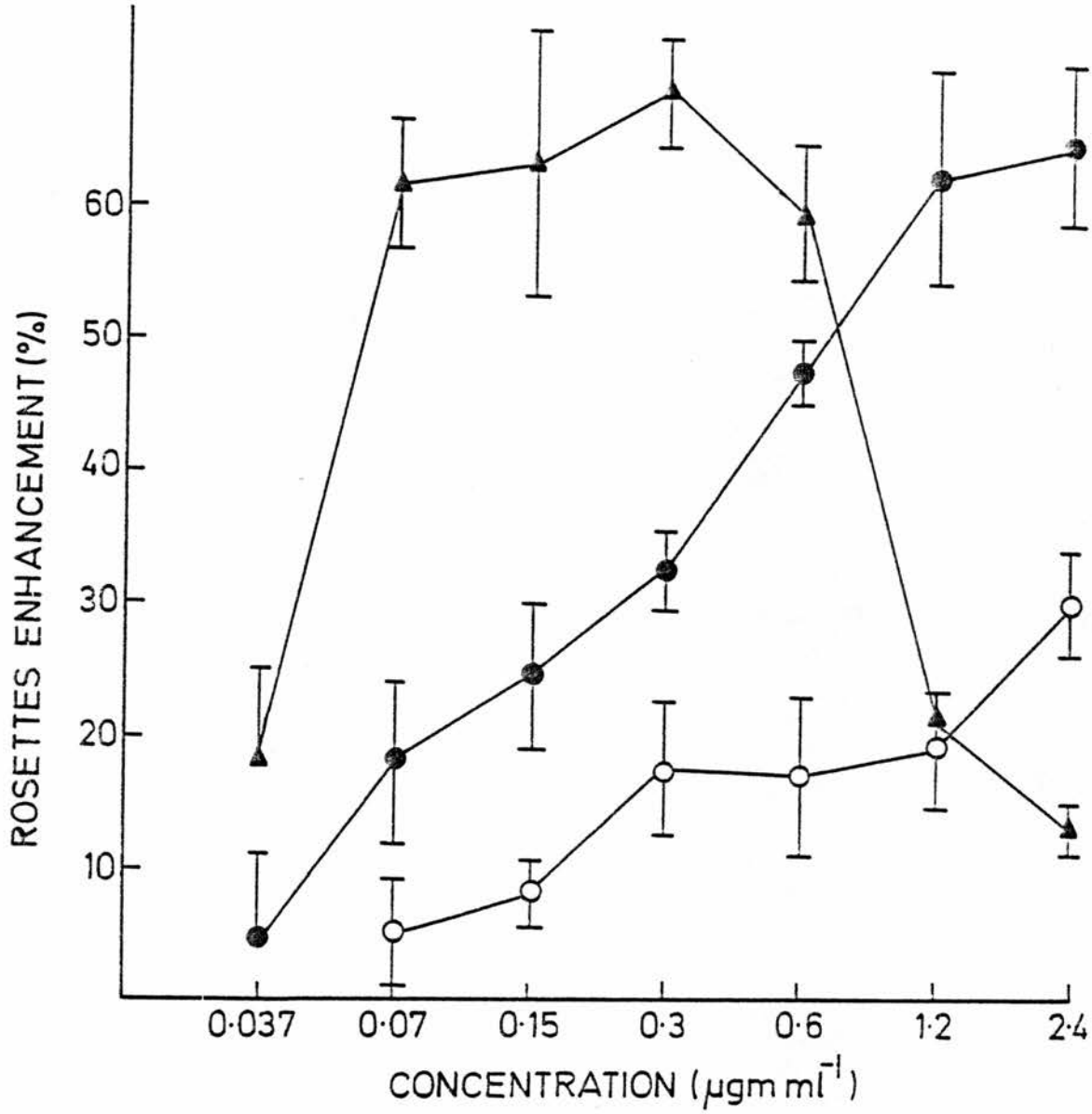


Figure 24

The effect of increasing concentrations of tobramycin on Fc receptor expression of alveolar macrophages. The macrophage monolayers were incubated for 30 minutes with antibiotic and washed prior to the addition of EA_G.

(▲——▲) incubated at 37°C assay 22°C

(●——●) incubated at 4°C assay 4°C

(○——○) incubated at 22°C assay 22°C

Each point represents the mean $\pm$ 1 SEM of three experiments.

Concentration $\mu gm\ ml^{-1}$	Added with bacteria to macrophages % Inhibition	Pre-treatment of macrophages % Enhancement
2.5	33.1 ± 6.4	3.1 ± 10.2
5	8.5 ± 10.4	10.4 ± 9.3
10	14.4 ± 10.3	19.2 ± 10.0
20	11.2 ± 11.2	17.1 ± 5.3
40	7.3 ± 8.5	12.3 ± 6.5
80	22.7 ± 4.0	7.3 ± 6.2
160	42.6 ± 13.7	17.3 ± 6.6

TABLE VI

The effect of ticarcillin on the binding of EA_G to rat alveolar macrophages. Each figure represents the mean ± 1 SEM of three experiments.

3.0 THE EFFECT OF TEMPERATURE ON TOBRAMYCIN-INDUCED ROSETTE ENHANCEMENT

Macrophage monolayers were incubated with tobramycin at 4°C and room temperature and the Fc rosette assay performed at the corresponding temperature. For the experiment performed at 4°C the erythrocytes were sensitized with a higher concentration of antibody to compensate for the decreased formation of rosettes at this temperature. The macrophages were incubated with tobramycin at 37°C as previously noted, but in this case the Fc receptor assay was performed at room temperature as phagocytosis occurs at 37°C.

At 4°C binding of EA_G was enhanced ($P < 0.05$) at concentrations above 1/4MIC and ($P < 0.01$) at 2 x MIC and 8 x MIC values (Figure 24). This effect was dose-dependent and reached a maximum of 64.5% at 8 x MIC. This maximum was similar to that obtained when the cells were incubated with tobramycin at 37°C and the assay performed at 22°C. However in this case a lower concentration of tobramycin was required to attain a similar result. The observed fall-off of enhancement in the 37°C experiment did not occur when the procedure was carried out at 4°C. However as higher concentrations were not studied, the possibility that enchancement may eventually drop at 4°C cannot be excluded.

When the experiment was carried out at room temperature enhancement was lower than that at 4°C ($P < 0.05$) and was only significant at 1/2MIC, 2 x MIC and 8 x MIC values. However the observed effect was dose-dependent and showed a similar pattern to that observed at 4°C.

Tobramycin slightly enhanced binding of EA_G to Fc receptors ($P < 0.05$ at MIC and 8 x MIC) when incubated with the macrophages at $4^{\circ}C$ prior to the assay being carried out at room temperature (Table VII).

Concentration $\mu gm\ ml^{-1}$	Binding EA_G % Enhancement
0.037	7.0 ± 7.3
0.075	12.0 ± 8.4
0.15	16.8 ± 8.3
0.3	22.5 ± 4.2*
0.6	15.2 ± 4.5
1.2	20.3 ± 9.2
2.4	28.9 ± 6.2*

*$p < 0.05$

TABLE VII

The effect of tobramycin on the binding of EA_G to alveolar macrophages. The macrophages were incubated with tobramycin at 4°C for 30 minutes prior to the assay which was performed at room temperature (22°C). Each figure represents the mean of three experiments ± 1 SEM.

4.0 TIME COURSE OF TOBRAMYCIN INDUCED ENHANCEMENT OF Fc RECEPTORS

Binding of EA_G to Fc receptors on rat alveolar macrophages was increased ($P<0.05$) when 1/2MIC value (0.15 ugm ml^{-1}) of tobramycin was incubated with macrophages for 10,20 and 30 minutes (Figure 25). However at 60 minutes, the level of binding had returned to control values and at 120 minutes, binding was decreased although not significantly.

Table VIII shows the effect of all concentrations of tobramycin when incubated with the macrophages for one and two hours prior to addition of the EA_G. Inhibition of binding was only significant ($P<0.05$) when macrophages were incubated for 2 hours with 8 x MIC of tobramycin. At one hour the enhancement was slight and the effect became inhibitory at 2 x MIC.

The inhibition of EA_G binding observed at two hours did not increase with longer times of pre-incubation (Table IX). However inhibition was significant ($P<0.05$) when 1/2MIC tobramycin was incubated with the macrophages for three hours.

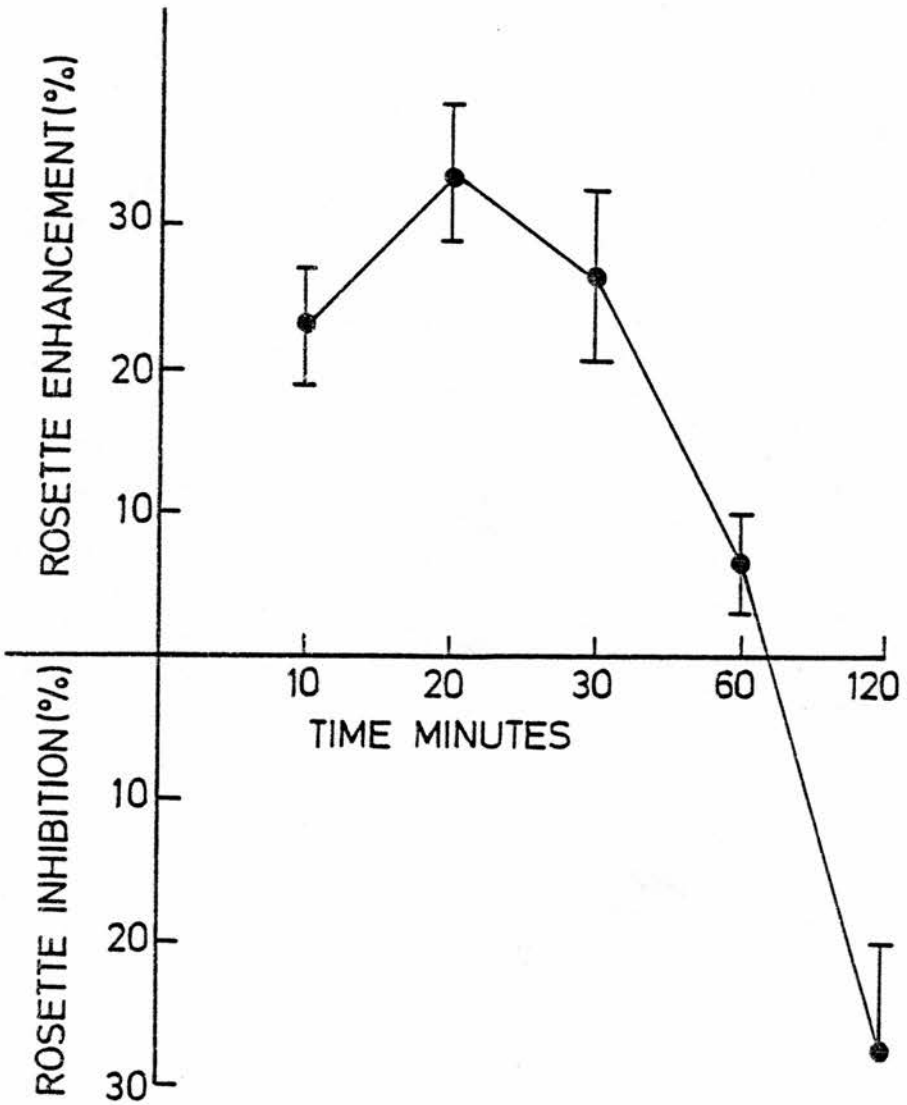


Figure 25

Time course of the effect of tobramycin on binding of EA_G to rat alveolar macrophages, after pre-incubation of the macrophages for 30 minutes with 1/2MIC value of tobramycin. Each point represents the mean $\pm$ 1SEM of three experiments.

Concentration $\mu gm\ ml^{-1}$	One Hour (%) Enhancement	Two Hours (%) Inhibition
0.037	11.5 ± 8.7	3.62 ± 11.8
0.07	27.5 ± 15.9	17.25 ± 12.0
0.15	9.4 ± 22.8	32.6 ± 8.4
0.3	5.7 ± 5.1	23.9 ± 6.1
0.6	10.1 ± 4.1 (-)	28.0 ± 9.2
1.2	1.95 ± 2.07 (-)	24.9 ± 11.8
2.4	4.2 ± 6.7 (-)	43.8 ± 8.0*

TABLE VIII

The effect of tobramycin on the binding of EA_G to rat alveolar macrophages. The macrophages were incubated with antibiotic for one or two hours prior to the assay. Each figure represents the mean ± 1 SEM of three experiments.

Time (Hours)	% Inhibition
2	25.4 ± 6.1
3	22.8 ± 4.1*
4	22.2 ± 6.2
5	20.4 ± 5.8

TABLE IX

Time course of the effect of tobramycin on the binding of EA_G to rat alveolar macrophages. The macrophages were incubated with 0.15 $\mu gm\ ml^{-1}$ tobramycin at various times prior to the Fc receptor assay. Each figure represents the mean ± 1 SEM of three experiments.

*($p < 0.05$)

5.0 THE EFFECT OF TOBRAMYCIN ON THE PHAGOCYTOSIS OF EA_G

Tobramycin caused a decrease in phagocytosis of bacteria yet an apparent increase in Fc receptor expression. This paradox was studied by investigating the effect of the antibiotic on the phagocytosis of EA_G. The macrophages were incubated for 30 minutes at 37°C with tobramycin and washed, then EA_G were added and incubated for a further 30 minutes at 37°C. The phagocytosis of EA_G was inhibited in a dose-dependent fashion and was significant ($P<0.05$) at values of 1/4MIC and above (Figure 26). Therefore these results are similar to those observed for the phagocytosis of P.aeruginosa and tobramycin appears to have a different effect on phagocytosis than on Fc receptor expression, as measured by the binding of EA_G to the macrophages.

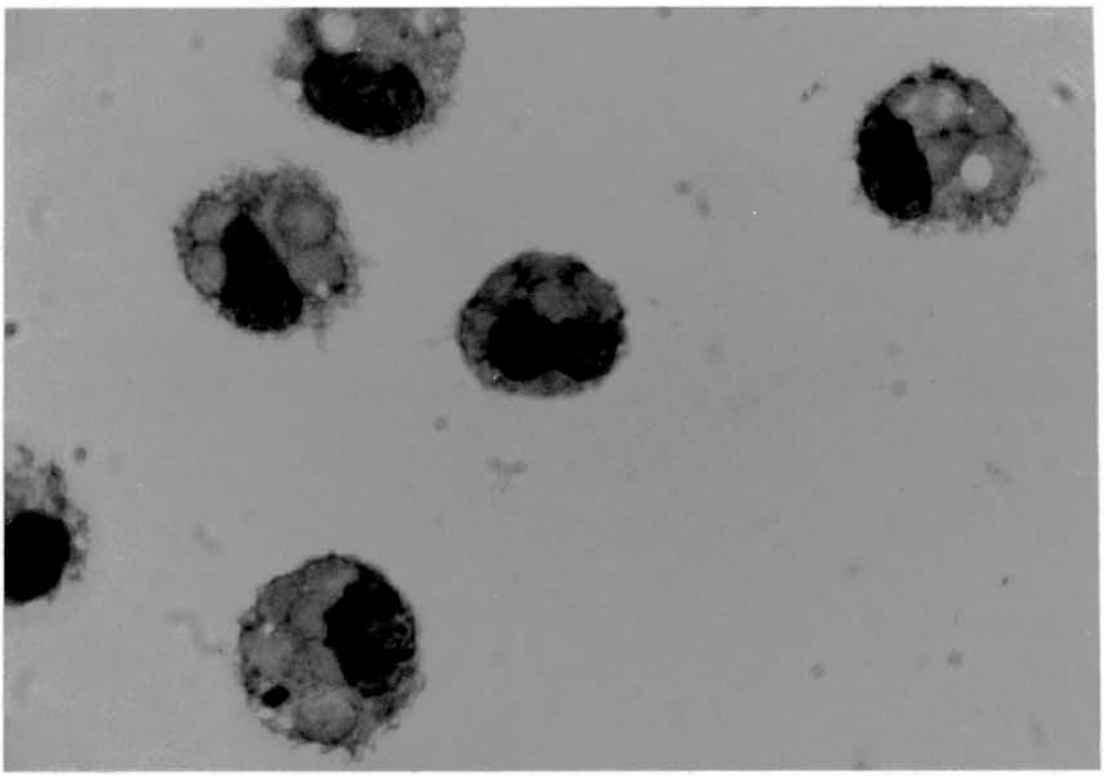


PLATE V

Phagocytosis of EA_G (prepared with mouse IgG2b monoclonal as the sensitising antibody) by rat alveolar macrophages. (May Grunwald/Giemsa, x 1,250 magnification).

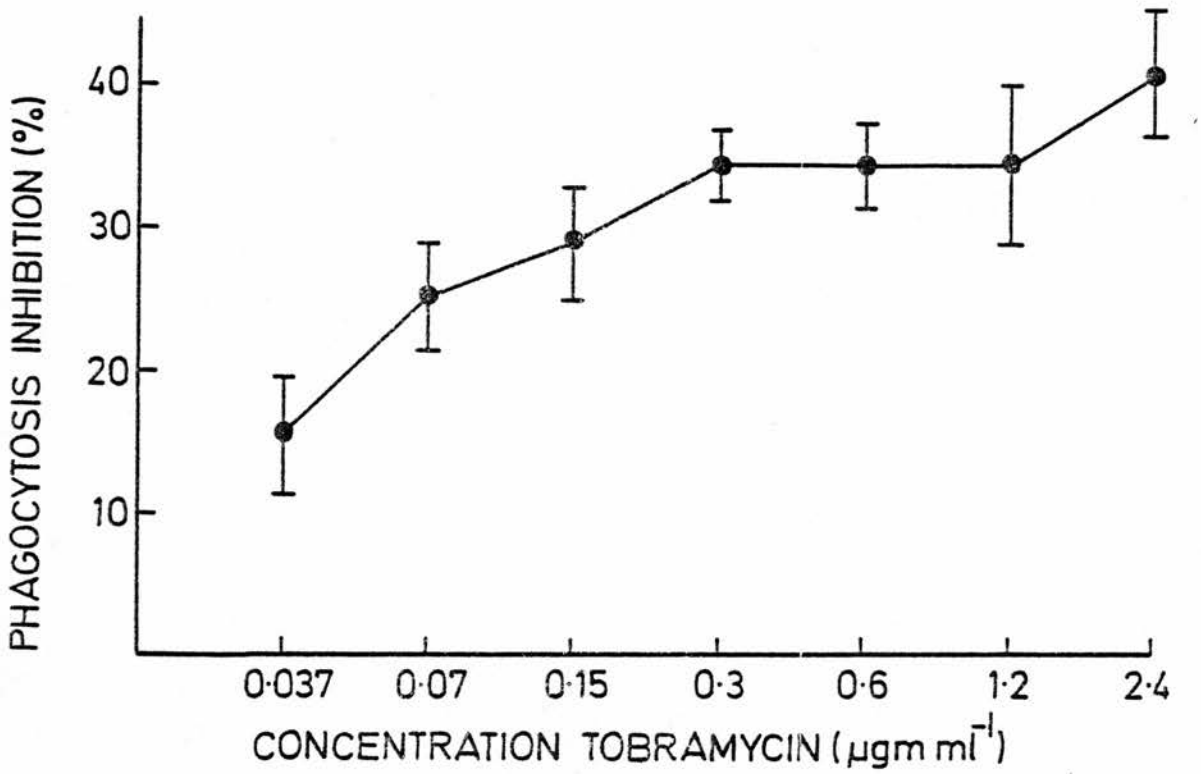


Figure 26

The effect of increasing concentrations of tobramycin on the phagocytosis of EA_G by rat alveolar macrophages. The macrophages were incubated with antibiotic for 30 minutes prior to the addition of the erythrocytes. Each point represents the mean $\pm$ 1SEM of three experiments.

6.0 SUMMARY

Tobramycin enhanced the binding of EA_G to rat alveolar macrophages when the phagocytes were pre-incubated with tobramycin for 30 minutes. This effect was dose, time and temperature dependent. Conversely pre-incubation of macrophages with tobramycin resulted in a decrease in the phagocytosis of sensitised erythrocytes. Azlocillin had no effect on Fc receptors when pre-incubated with the macrophages.

Binding of EA_G to Fc receptors on the macrophages was inhibited in a dose-dependent fashion when either tobramycin or azlocillin was co-incubated with the macrophage monolayers and erythrocytes.

Ticarcillin had no effect on EA_G binding with either procedure.

SECTION III

THE EFFECT OF TOBRAMYCIN AND AZOLCILLIN ON P.AERUGINOSA AND SENSITIZED SHEEP ERYTHROCYTES

1.0 INTRODUCTION

It was shown in the previous section that tobramycin and azlocillin had an inhibitory effect on phagocytosis and Fc receptor expression when added with the opsonised bacteria or EA_G (indicator cells) to the macrophage monolayers. Therefore these effects may be a result of the action of the antibiotic on the indicator cells as opposed to a direct effect on the macrophages. To examine this possibility, these two antibiotics were incubated with opsonised bacteria or sensitised sheep erythrocytes for 30 minutes and the cells washed prior to their addition to the macrophage monolayers. The effect of tobramycin on the opsonisation procedure was also evaluated.

2.0 THE EFFECT OF TOBRAMYCIN ON THE INDICATOR CELLS AND OPSONISATION

Tobramycin did not alter the phagocytosis of opsonised P.aeruginosa or the binding of EA_G when incubated with these cells (Table X). However the phagocytosis of EA_G was enhanced after tobramycin treatment of the sheep erythrocytes (Figure 27). This effect was significant ($P < 0.05$) at 1/8MIC, 2 x MIC and 4 x MIC values, and ($P < 0.01$) at 4 x MIC value. Tobramycin was also added to the antibody and bacterial mixture during opsonisation, the cells washed and added to the macrophages. After this treatment there was no difference in the subsequent phagocytosis of the bacteria as compared to untreated bacteria (Table XI). Therefore tobramycin has no effect on the opsonisation procedure as measured by subsequent phagocytosis.

Concentration $\mu gm\ ml^{-1}$	Phagocytosis of bacteria % Inhibition	Binding of EA_G % Inhibition
0.037	8.5 ± 9.1	11.4 ± 4.2 (-)
0.075	3.3 ± 14.8	18.4 ± 5.6 (-)
0.15	2.4 ± 5.7	5.2 ± 2.4
0.3	3.0 ± 12.3	28.1 ± 10.2
0.6	4.2 ± 6.1	1.4 ± 4.2
1.2	10.2 ± 4.1	6.6 ± 3.6
2.4		4.8 ± 2.2

TABLE X

The effect of tobramycin on the indicator cells. The opsonised bacteria or EA_G were incubated for 30 minutes at 37°C with tobramycin and washed before addition to the alveolar macrophages. Each figure represents the mean ± 1 SEM of three experiments.

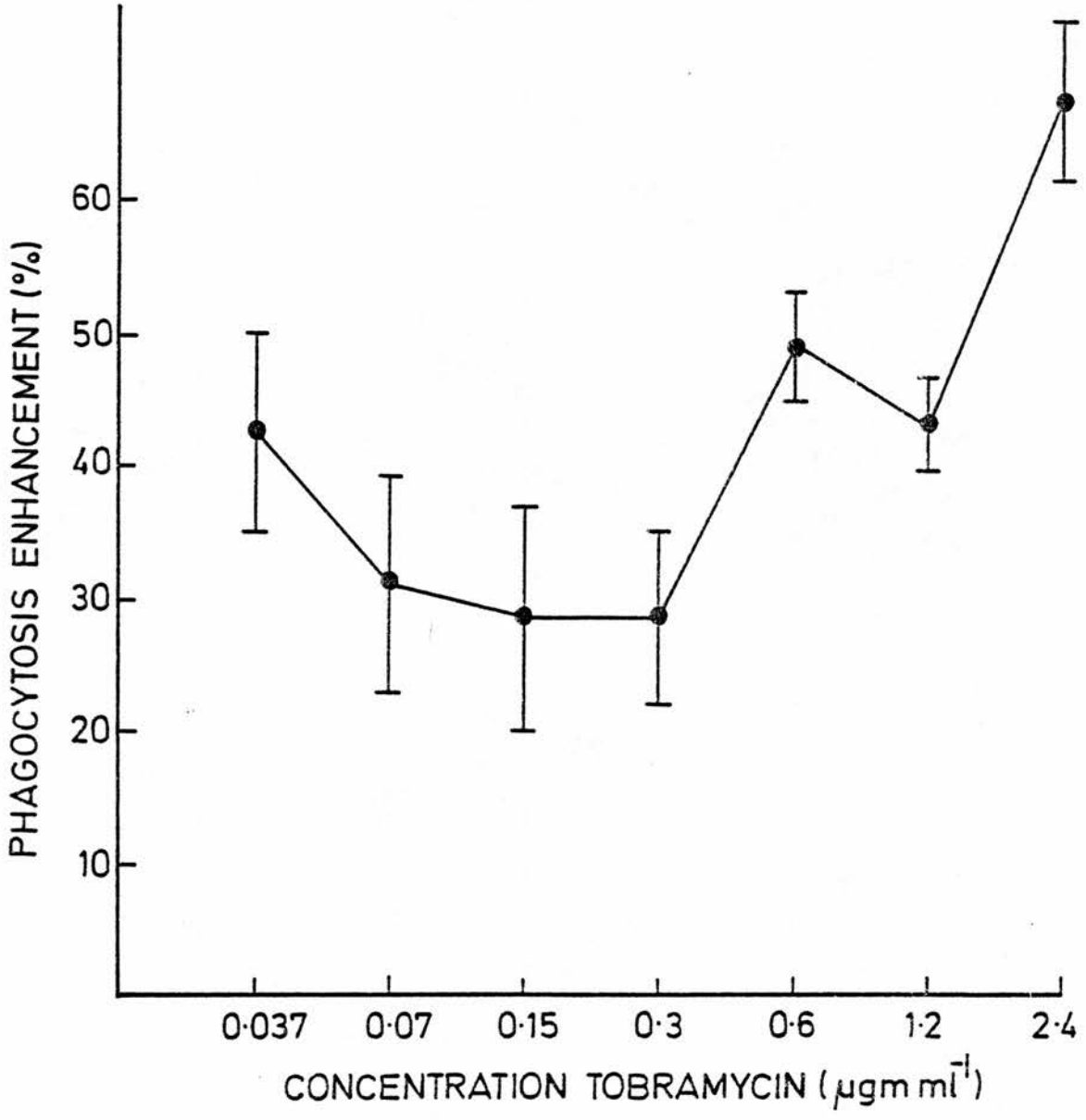


Figure 27

The effect of increasing concentrations of tobramycin on the phagocytosis of EA_G. The sensitised sheep erythrocytes were incubated with the antibiotic for 30 minutes, and washed prior to addition to the macrophage monolayer. Each point represents the mean $\pm$ 1 SEM of three experiments.

Concentration $\mu gm\ ml^{-1}$	Phagocytosis % Inhibition
0.037	6.4 ± 11.1
0.07	0.5 ± 9.9 (-)
0.15	8.8 ± 8.7
0.3	12.5 ± 10.2
0.6	4.9 ± 6.4
1.2	15.4 ± 7.0
2.4	13.1 ± 9.7

TABLE XI

The effect of tobramycin on opsonisation. Tobramycin was added to the bacteria and serum mixture during opsonisation. Each figure represents the mean ± 1 SEM of four experiments.

3.0 THE EFFECT OF AZLOCILLIN ON THE INDICATOR CELLS

Azlocillin had no direct effect on the indicator cells as the phagocytosis of P.aeruginosa or the binding of EA_G was unaltered after incubation with the antibiotic (Table XII). Therefore, although the continual presence of this antibiotic appears to be required for inhibition of phagocytosis and Fc receptor expression to occur, the observed effects are not a result of any direct action of azlocillin on the bacteria or erythrocytes.

Concentration $\mu gm\ ml^{-1}$	Phagocytosis of bacteria % Inhibition	Binding of EA_G % Inhibition
0.6	5.5 ± 10.1	15.2 ± 2.4
1.2	2.5 ± 6.2	11.5 ± 3.5
2.5	2.5 ± 7.5	16.1 ± 10.2
5.0	3.5 ± 8.1	14.2 ± 2.4
10.0	7.1 ± 1.6	3.5 ± 3.6
20.0	9.2 ± 6.5	5.1 ± 6.3
40.0	10.1 ± 4.2	8.4 ± 5.2

TABLE XII

The effect of azlocillin on the indicator cells. The opsonised bacteria or EA_G were incubated for 30 minutes at 37°C with azlocillin and washed before addition to the alveolar macrophages. Each figure represents the mean ± 1 SEM of three experiments.

4.0 SUMMARY

Tobramycin and azlocillin had no direct effect on either opsonised P.aeruginosa or sensitised sheep erythrocytes as measured by their subsequent phagocytosis or binding. However sensitised sheep erythrocytes became more susceptible to phagocytosis after treatment with tobramycin for 30 minutes. Tobramycin had no effect on the opsonisation of P.aeruginosa by immune serum as measured by the subsequent phagocytosis of the bacteria by the alveolar macrophages.

SECTION IV

STUDIES ON THE MECHANISM OF ACTION OF Fc RECEPTOR MODULATION BY TOBRAMYCIN

1.0 INTRODUCTION

Tobramycin was shown to enhance Fc receptor expression, when pre-incubated with the macrophages for 30 minutes, but enhancement no longer occurred if macrophages were incubated for 60 minutes or longer with the antibiotic. It therefore seemed possible that Fc receptors were being shed from the macrophage membrane as a result of the tobramycin treatment. This possibility was investigated by taking the supernatants from tobramycin treated macrophage monolayers and examining their effect on EA_G and freshly prepared macrophage monolayers. Electrophoretic separation of the supernatants on polyacrylamide gel was also performed.

2.0 THE EFFECT OF SUPERNATANTS ON EA_G

Supernatants were taken from macrophages that had been treated with tobramycin for one hour and prepared as discribed in Materials and Methods. The supernatants were made up to the original volume and mixed in a 1:1 or 2:1 ratio with the EA_G for 30 minutes at 37°C. The erythrocytes were then washed and added to fresh macrophage monolayers and an Fc receptor assay performed. The binding of the treated EA_G was decreased in a dose-dependent fashion (Figure 28). The effect was greater ($P < 0.05$) at the higher supernatant: EA_G ratio of 2:1, where inhibition reached a maximum of 58% at 4 x MIC value of tobramycin and was significant ($P < 0.01$) at MIC - 8 x MIC values.

When the supernatants were taken from macrophage monolayers that had been incubated for two hours, binding of the EA_G was decreased to a lesser extent than supernatants obtained at one hour. However this decrease was significant ($P < 0.05$) at 1/8MIC, 4 x MIC and 8 x MIC and ($P < 0.01$) at MIC and 2 x MIC values, reaching a maximum of 37.5% inhibition at the highest concentration used (Figure 29).

Supernatants taken from macrophages that had been incubated for 30 minutes with tobramycin had no effect on the binding of EA_G (Table XIII).

Dialysis of the supernatants removed any tobramycin that might have been present as at the highest concentration used, there was no inhibition of growth of *P.aeruginosa* when cultured with the prepared supernatants. Therefore the results are not attributable to the possible presence of tobramycin in the supernatant.

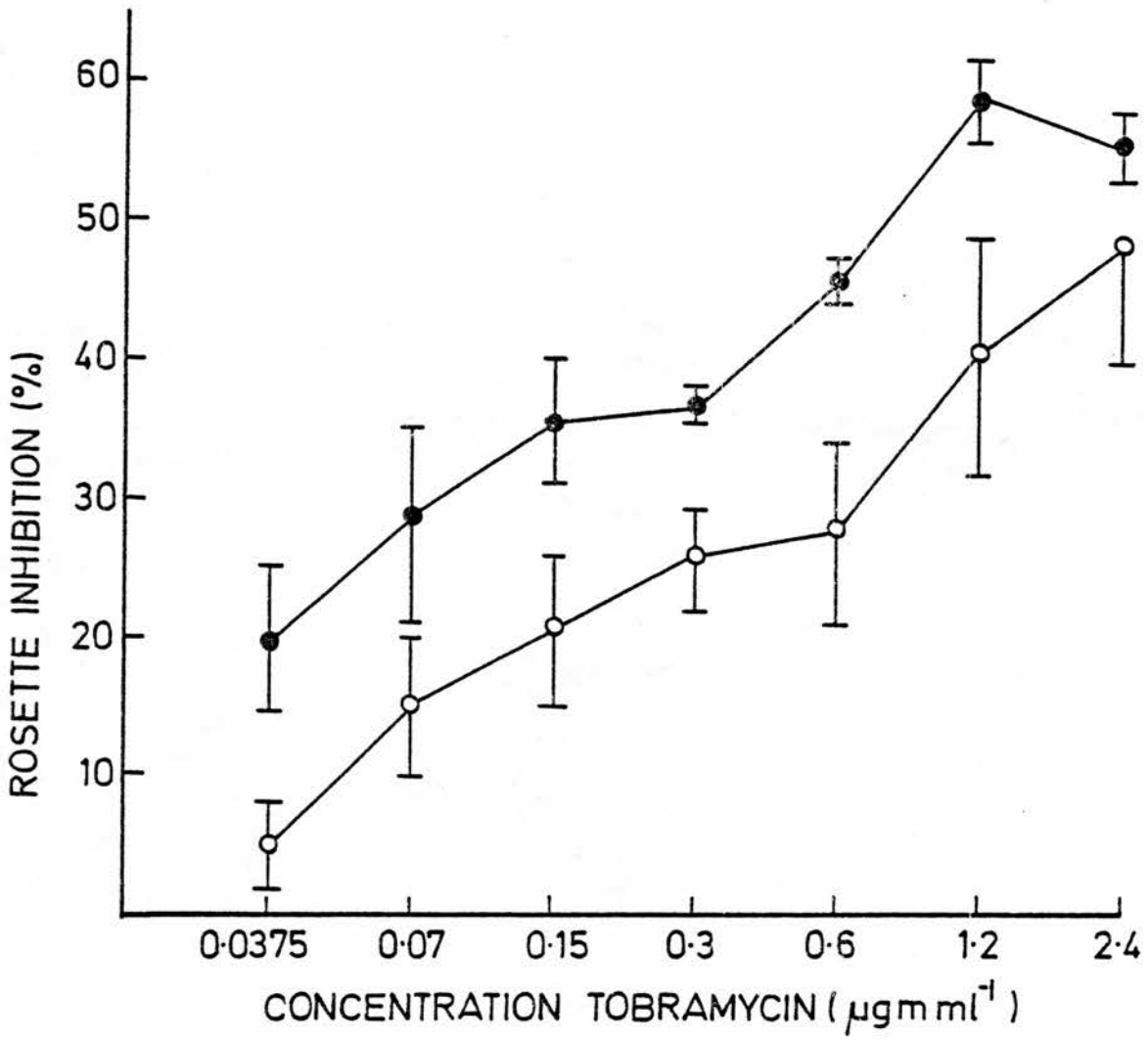


Figure 28

The effect of supernatants taken from macrophages incubated with tobramycin for one hour, on the binding of EA_G to alveolar macrophages. Supernatants were incubated with EA_G for 30 minutes at a 1:1 ratio (O——O) or 2:1 ratio (●——●) prior to the Fc receptor assay. Each point represents the mean $\pm$ 1 SEM of four experiments.

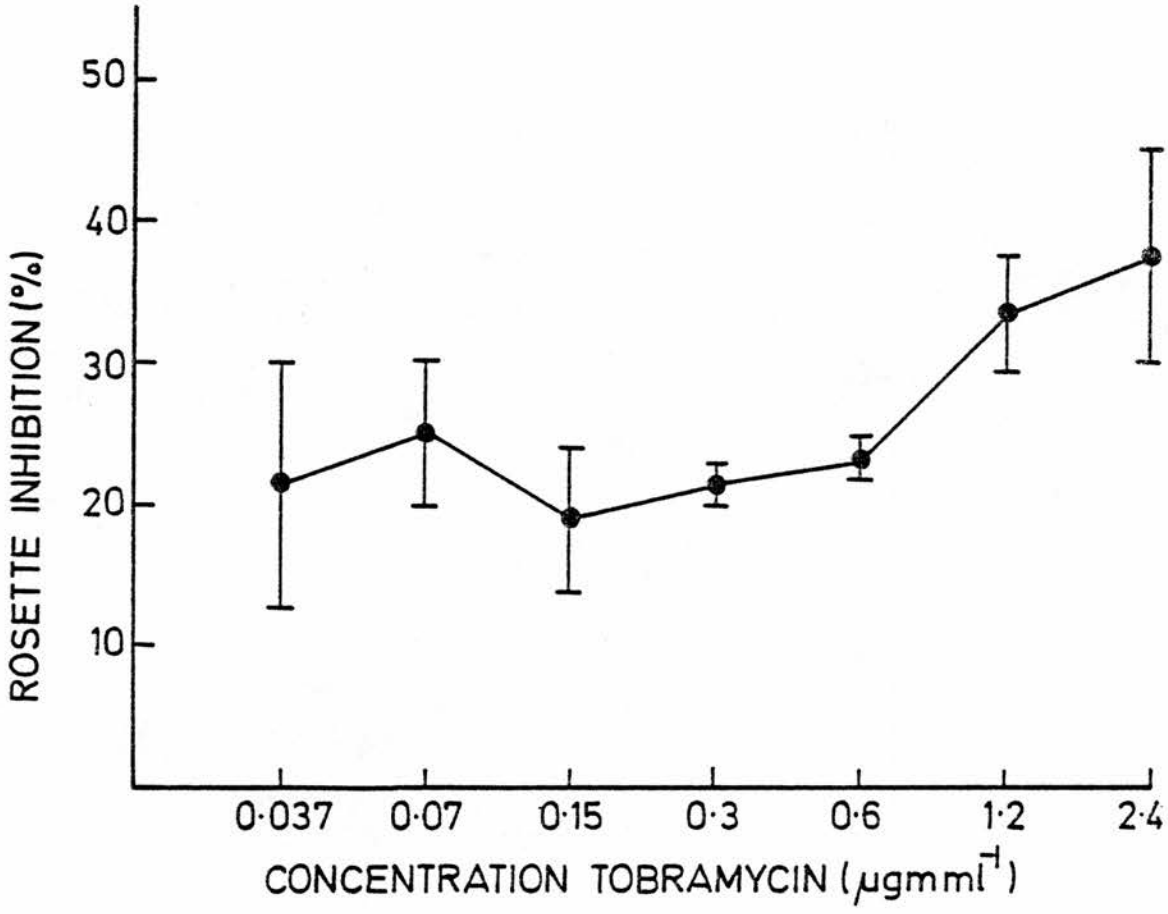


Figure 29

The effect of supernatants taken from macrophages incubated with tobramycin for two hours, on the binding of EA_G to rat alveolar macrophages. Procedure as described for Figure 28 and the ratio of supernatant to red cells was 1:1. Each point represents the mean $\pm$ 1 SEM of four experiments.

Concentration $\mu gm\ ml^{-1}$	Binding of treated EA_G % Enhancement
0.037	7.0 ± 3.2 (-)
0.07	8.3 ± 5.7 (-)
0.15	2.0 ± 4.0
0.3	6.9 ± 12.2
0.6	15.8 ± 7.1
1.2	19.3 ± 8.0
2.4	16.6 ± 6.2

TABLE XIII

The effect of supernatants from macrophages treated with tobramycin for 30 minutes, on the binding of EA_G to alveolar macrophages. Supernatants were incubated with EA_G for 30 minutes at a 3:1 ratio. Each figure represents the mean ± 1 SEM of four experiments.

3.0 THE EFFECT OF SUPERNATANTS ON ALVEOLAR MACROPHAGES

Supernatants, prepared as previously described were incubated with fresh alveolar macrophage monolayers for one hour at room temperature and the cells washed, prior to performing an Fc receptor assay. The binding of EA_G to the macrophages was increased, ($P < 0.05$) at 1/8MIC, ($P < 0.01$) at MIC, 8 x MIC, 16 x MIC, after incubation with supernatant reconstituted to original volume (Figure 30). When the supernatant was diluted 1:5, binding was increased, but to a lesser extent ($P < 0.05$), showing a concentration effect. At this dilution enhancement of rosette formation was significant ($P < 0.05$) at 8 x MIC and ($P < 0.01$) at the MIC and 16 x MIC values. With undiluted supernatant the maximum response (77%) was reached at 8 x MIC value.

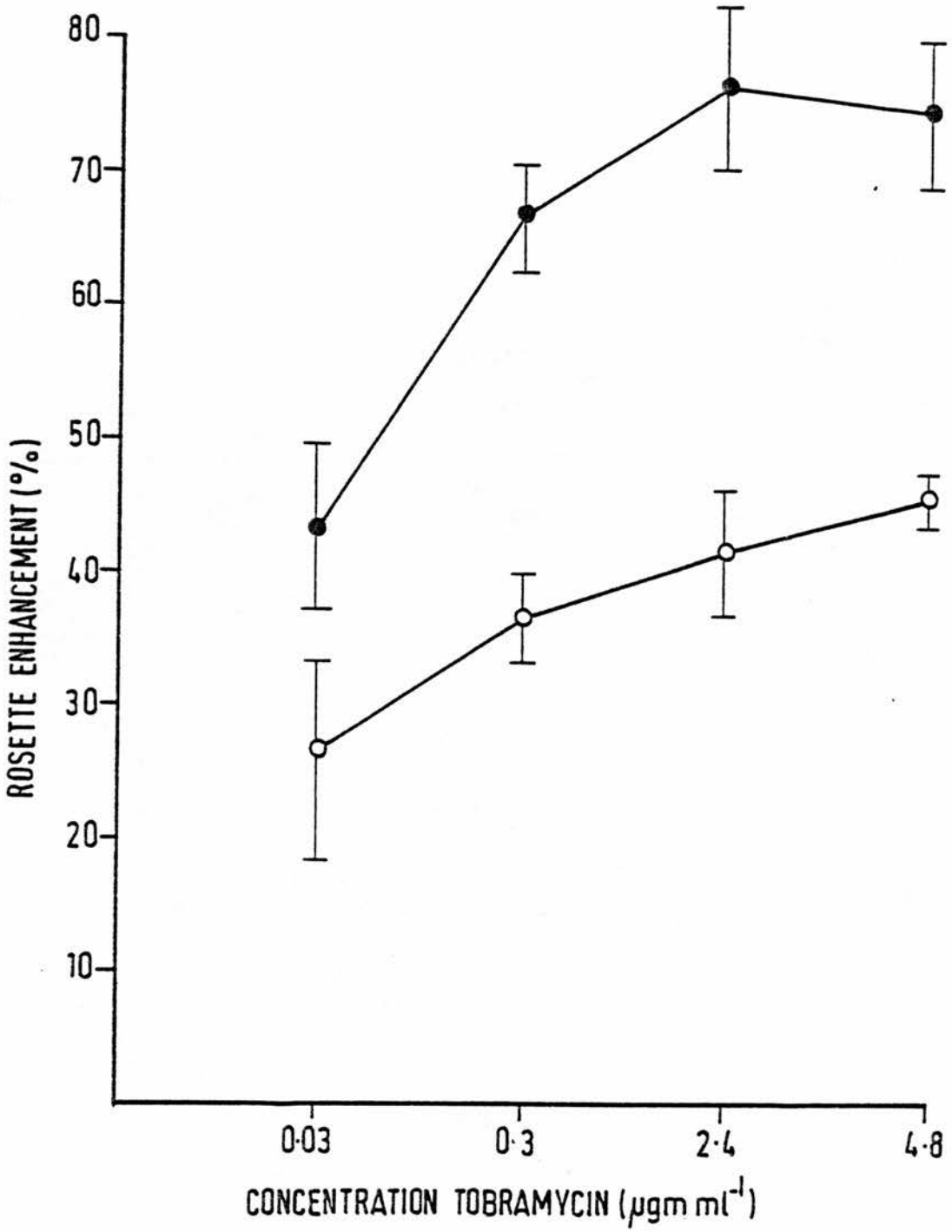


Figure 30

The effect of supernatant taken from macrophages incubated with tobramycin for one hour on alveolar macrophages. The macrophages were incubated with supernatant for one hour and washed prior to the addition of the EA_G.

(●——●) dialysate reconstituted in original volume

(○——○) 1/5 dilution

Each point represents the mean ± 1 SEM of three experiments.

4.0 THE EFFECT OF SUPERNATANTS ON THE BINDING OF STAPH ALBUS

To check the specificity of the effects that the supernatants had on EA_G and macrophages, Staph.albus were incubated with supernatants for 30 minutes at 37°C, washed and added to macrophage monolayers. There was no observed effect on the binding of bacteria that had been treated (Table XIV) indicating the presence of a factor in the supernatants with a specificity for antibody sensitised erythrocytes.

Concentration tobramycin μgm ml^{-1}	% Inhibition
0.03	7.4 ± 4.2
0.15	1.7 ± 3.6
0.3	2.7 ± 2.5
1.2	2.9 ± 3.0
2.4	3.05 ± 4.1

TABLE XIV

The effect of supernatants on the binding of Staph.albus to rat alveolar macrophages. The bacteria were incubated with supernatants at 37°C for 30 minutes, washed and added to the monolayers. Each figure represents the mean ± 1 SEM of three experiments.

5.0 THE EFFECT OF SUPERNATANTS ON THE AGGLUTINATION OF SENSITISED ERYTHROCYTES

Concentrated supernatants from tobramycin treated macrophages (10ml supernatant reconstituted in one ml HBSS after freeze-drying) caused the agglutination of EA_G in V bottomed microtitre plates. Agglutination occurred until a dilution of 1/8 was reached. Control supernatants did not show this effect at the concentrations studied.

Supernatants that had been absorbed with formalised EA_G, were no longer capable of agglutinating fresh EA_G.

6.0 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The supernatants from tobramycin treated macrophages inhibited the binding of EA_G on incubation with the red cells and enhanced EA_G binding on incubation with the macrophages, but had no effect on bacterial binding. Therefore it seemed likely that the supernatants contained a blocking factor that bound to red cells or sensitising antibody and inhibited the attachment of the cells to the macrophage Fc receptor. Conversely the factor would bind to the macrophage membrane and cause enhanced binding of EA_G. PAGE was performed on supernatants from treated and untreated macrophages to examine the possibility that a blocking factor was present in the treated supernatants.

Plate VI shows a PAGE gel of the supernatants that had been prepared as described in Materials and Methods, 100µl of each sample was loaded onto the gel. The supernatant from macrophages treated with 2.4µgml^{-1} tobramycin contained more material, especially at two bands approximating to 66,000 and 45,000 M.Wt. The control which contained medium alone and medium with tobramycin showed very faint bands, so that the observed effect was not due to the tobramycin present in the supernatant, but protein released from the macrophage monolayers.

Plate VII shows a repeat of this experiment and again the supernatant from 2.4µgml^{-1} tobramycin treated macrophages contains more protein than the control supernatant, especially at two bands. This indicates that the first result was not a "loading"effect . The molecular weight marker was over-diluted in this experiment so

that the bands are too faint to be seen.

Sensitised erythrocytes were also treated with supernatants, washed and the resulting supernatant studied on PAGE. This experiment was performed to determine whether the EA_G bound a factor contained in the supernatant. If this occurred the bands containing more material with supernatant alone, would be removed in incubation with EA_G. However from Plate VII it would appear that the red cells were lysed during the procedure so that conclusions cannot be drawn. Tobramycin supernatants do, though appear to cause more damage to the red cells than control supernatants. In this experiment the supernatants were not dialysed prior to incubation with the EA_G, so that it is possible that the antibiotic may have a direct effect on the red cells. It was previously shown that tobramycin treatment of EA_G caused an increase in the phagocytosis of the erythrocytes (Figure 27).

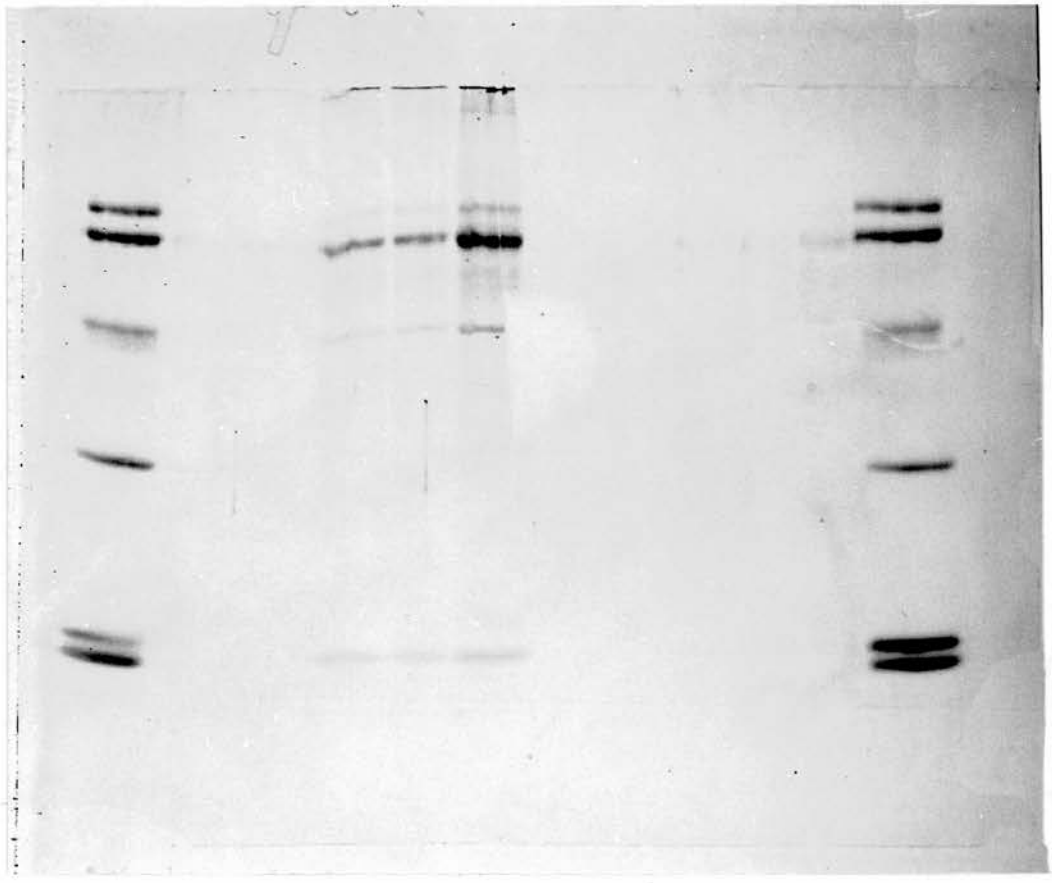


Plate VI

Polyacrylamide gel eletrophoresis of supernatants taken from untreated (control) and tobramycin treated macrophages monolayers after one hour incubation at 37^{0}C.

a = molecular wt marker:- 12,300, 17,200, 25,700, 45,000, 66,200, 76-78,000.

b = Eagles MEM alone

c = Eagles MEM containing 2.4 µgm ml^{-1} tobramycin

d = Control supernatant

e = Supernatant from macrophages treated with 0.3 µgm ml^{-1} tobramycin

f = Supernatant from macrophages treated with 2.4 µgm ml^{-1} tobramycin

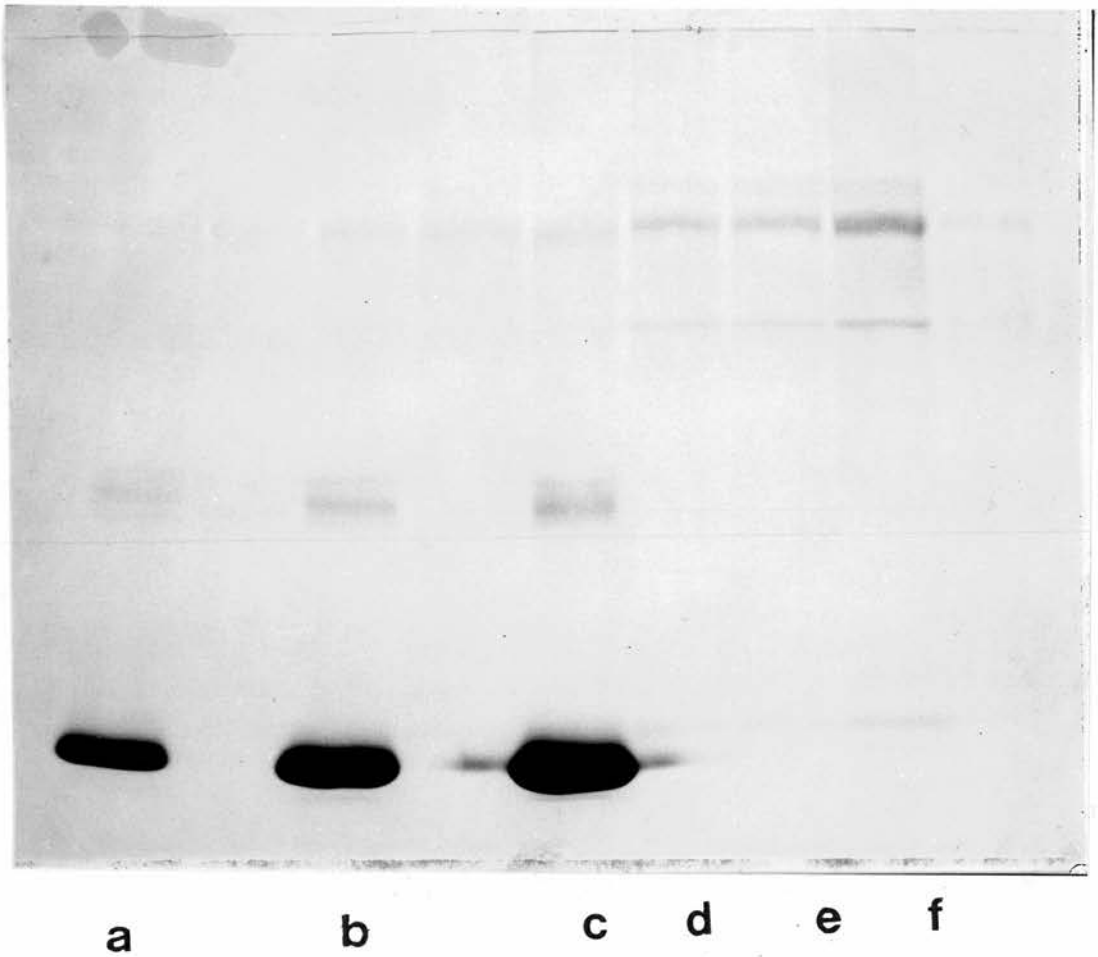


Plate VII

Polyacrylamide gel electrophoresis of supernatants taken from EA_G incubated with macrophage supernatant for 30 minutes at 37°C and of supernatants from macrophages described in Plate VI.

a = EA_G incubated with control supernatant

b = EA_G incubated with 0.3 µgm ml^{-1} tobramycin supernatant

c = EA_G incubated with 2.4 µgm ml^{-1} tobramycin supernatant

d = control supernatant

e = 0.3 µgm ml^{-1} supernatant

f = 2.4 µgm ml^{-1} supernatant

7.0 SUMMARY

Supernatants taken from tobramycin treated macrophages inhibited the binding of EA_G to Fc receptors, when pre-incubated with the EA_G and enhanced the binding of EA_G to Fc receptors when pre-incubated with the macrophages. The supernatants had no effect on the binding of Staph.albus to macrophages when incubated with the bacteria. These results indicated the presence of a factor in the supernatants that possibly bound to the antibody on the red cells to inhibit attachment to the macrophage Fc receptor. Furthermore, the tobramycin supernatants caused the agglutination of EA_G. This activity was lost after incubation with formalised EA_G. Polyacrylamide gel electrophoresis revealed that more protein is released from tobramycin treated macrophages, especially at two bands that may correspond to the Fc receptor.

Supernatants containing tobramycin caused more damage to sensitised erythrocytes than control supernatants.

SECTION V

THE EFFECT OF TOBRAMYCIN, AZLOCILLIN AND TICARCILLIN ON Fc RECEPTORS ON HUMAN PERIPHERAL BLOOD MONOCYTES

1.0 INTRODUCTION

In the previous sections, tobramycin and azlocillin were shown to have an effect on the Fc receptor expression of rat alveolar macrophages. These results indicated the necessity for studies on human cells, for only then could any clinical relevance of these effects be determined. Alveolar macrophages from healthy volunteers and patients are difficult to obtain, especially in a non-clinical setting. Peripheral blood monocytes are the precursor cells of alveolar macrophages (Thomas et al., 1976), and are readily and easily obtained. Therefore some studies were carried out on the effect of the three antibiotics, although mainly tobramycin, on the binding of IgG2b sensitised sheep erythrocytes to human peripheral blood monocytes.

2.0 THE EFFECT OF TOBRAMYCIN ON Fc RECEPTORS

Tobramycin inhibited the binding of EA_G to human peripheral blood monocytes when the antibiotic was added with the erythrocytes to the monolayers. Inhibition occurred when results from six different individuals were pooled , ($P<0.05$) at 1/4MIC, 1/2MIC and MIC values (Figure 31) and the curve obtained was similar to that observed when rat alveolar macrophages were studied. Inhibition of binding was also observed ($P<0.05$) at 1/4MIC to 2 x MIC values when monocytes taken from the same individual on different days were studied (Figure 31).

When the monocytes were pre-incubated with tobramycin for 30 minutes prior to the addition of EA_G, there was no effect on Fc receptors when results from different individuals were pooled (Table XV). This may be a result of individual variation, for when cells were taken from a single individual and studied on different days, tobramycin treatment caused a decrease ($P<0.05$) (Figure 32) or an increase (Figure 33) in the binding of EA_G to the monocytes. In this case a longitudinal study may reveal effects that are masked when results from many individuals are pooled.

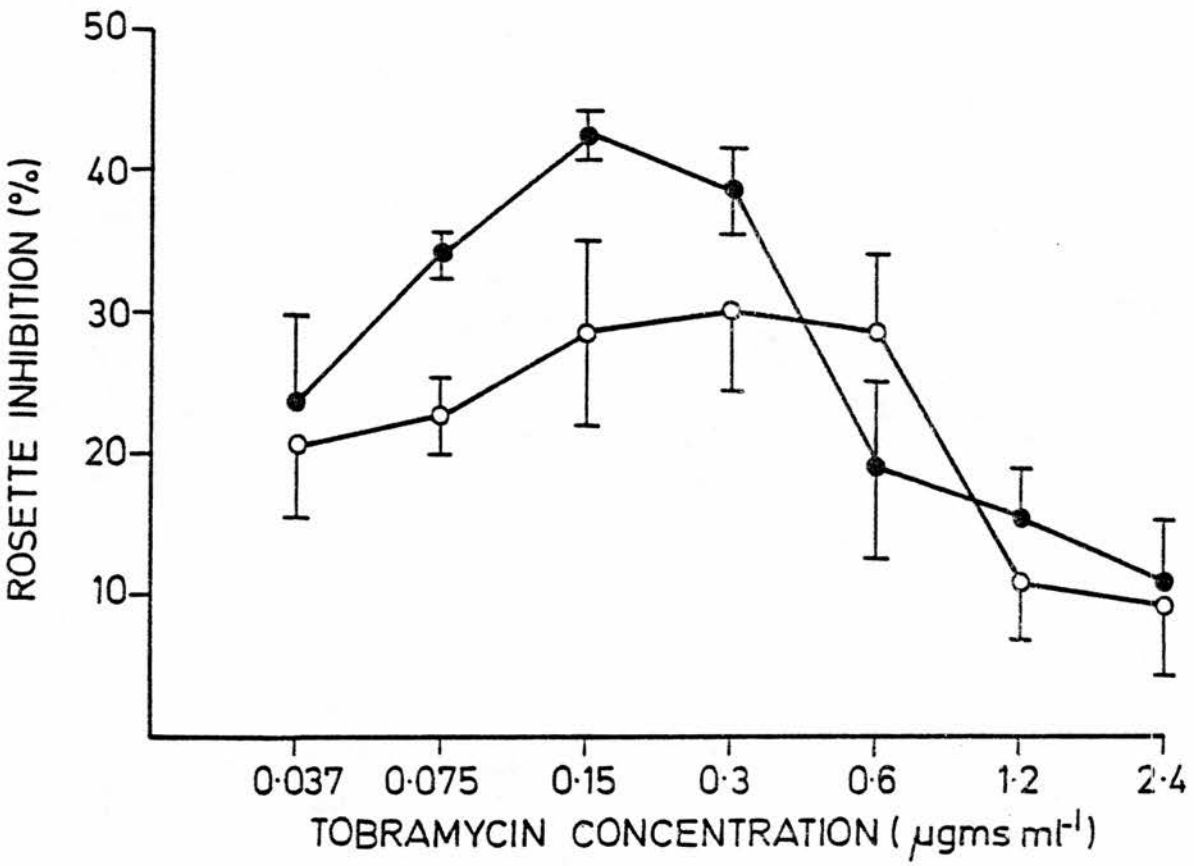


Figure 31

The effect of increasing concentrations of tobramycin on binding of EA_G to human monocytes, when the antibiotic was added with the erythrocytes.

(●——●) results pooled from six different individuals

(○——○) results from monocytes taken at different days from the same individual

Each point represents the mean ± 1 SEM of six experiments (●——●) or four experiments (○——○).

Concentration $\mu gm\ ml^{-1}$	Binding EA_G % Enhancement
0.037	0.16 ± 11.6
0.07	7.0 ± 11.9
0.15	10.1 ± 10.1
0.3	4.5 ± 11.3
0.6	16.7 ± 16.9
1.2	8.5 ± 8.9
2.4	4.8 ± 12.3

TABLE XV

The effect of tobramycin on human monocytes. Monocyte monolayers were incubated with antibiotic for 30 minutes. Each figure represents the mean of five experiments (each with a different subject) ± 1 SEM.

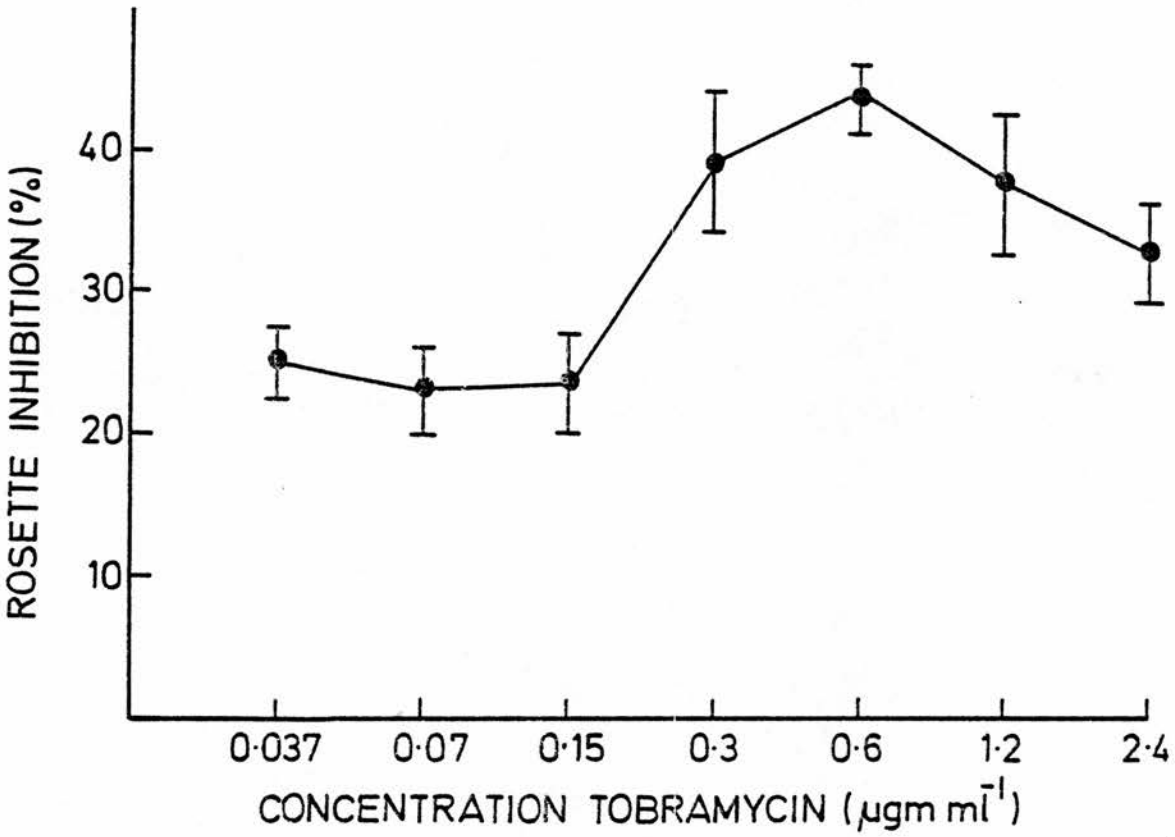


Figure 32

The effect of increasing concentrations of tobramycin on the binding of EA_G to human monocytes taken from one individual. The monocytes were pre-incubated with antibiotic for 30 minutes and washed prior to the addition of the erythrocytes. Each point represents the mean $\pm$ 1 SEM of three experiments.

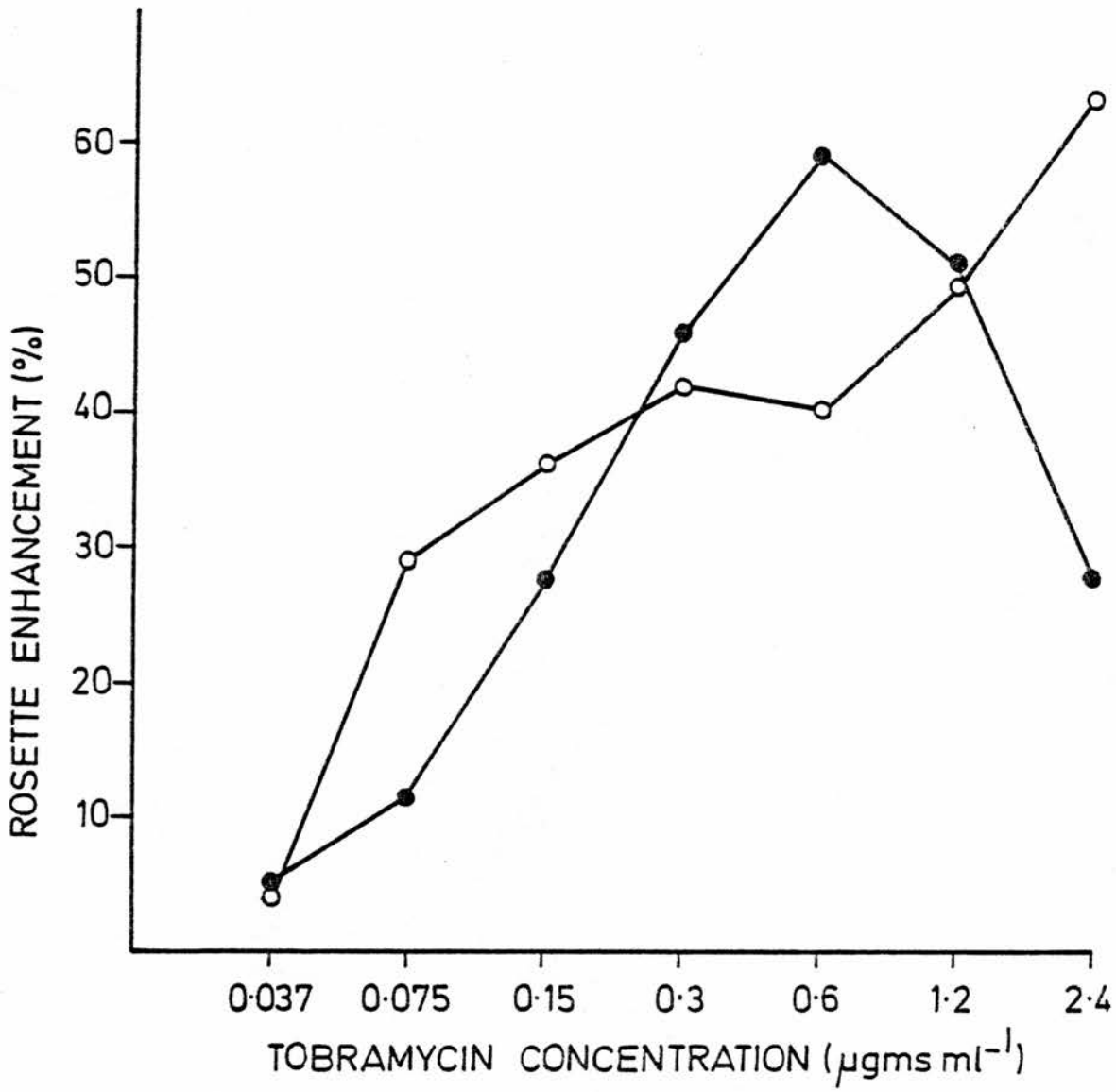


Figure 33

The effect of increasing concentrations of tobramycin on the binding of EA_G to human monocytes. The monocytes were incubated for 30 minutes with antibiotic prior to the addition of the erythrocytes. Results from two individuals shown. Each point represents the mean of two experiments.

3.0 THE EFFECT OF AZLOCILLIN AND TICARCILLIN ON Fc RECEPTORS

When azlocillin was added to monocyte monolayers with the EA_G, binding was enhanced when monocytes were taken from the same individual (Figure 34). However this experiment was only repeated twice, so that any significant difference could not be estimated. Ticarcillin had no effect on the binding of EA_G to monocyte Fc receptors (Table XVI).

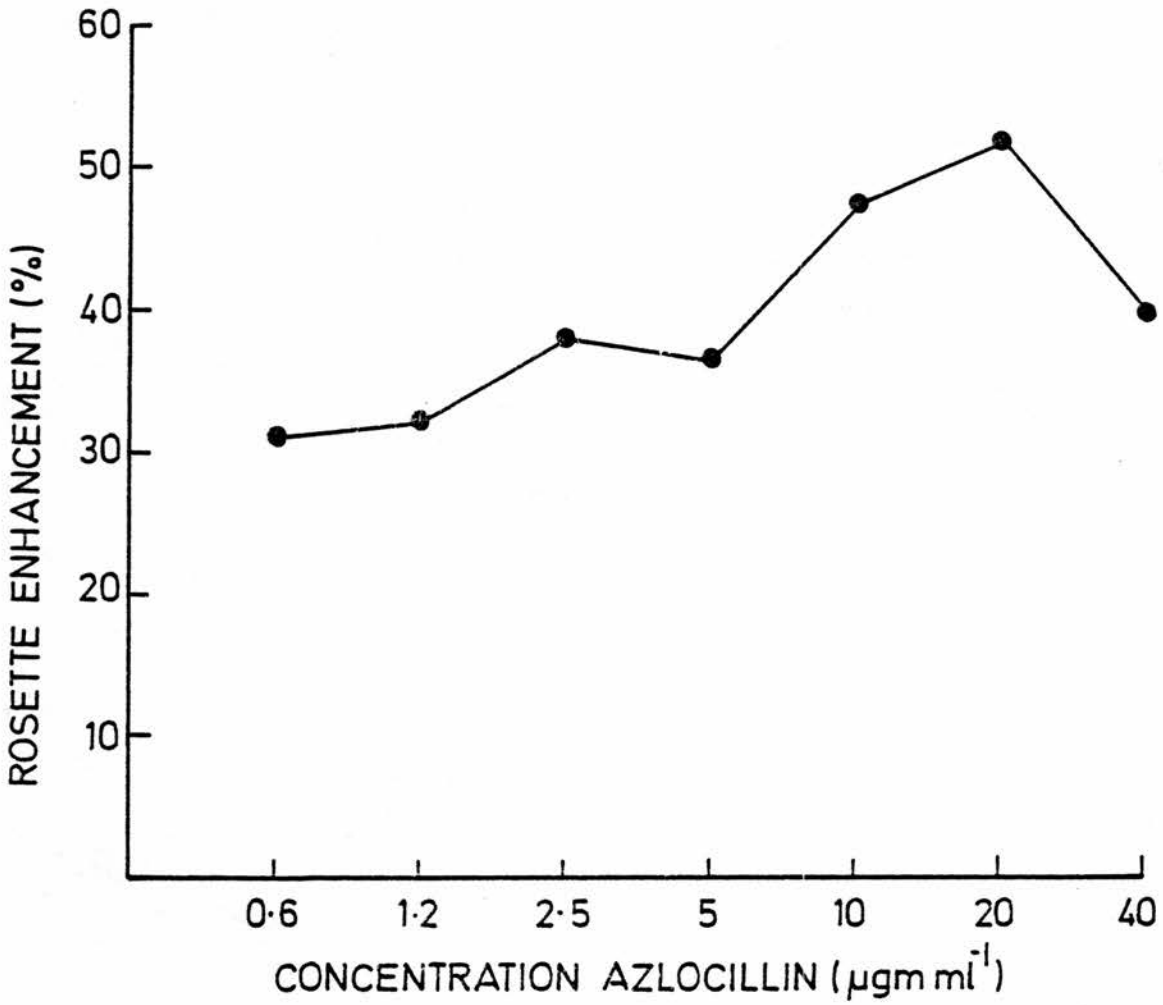


Figure 34

The effect of increasing concentrations of azlocillin on the binding of EA_G to human monocytes, taken from the same individual. The antibiotic was added with the erythrocytes to the monocyte monolayers. Each point represents the mean of two experiments.

Concentration $\mu gm\ ml^{-1}$	Binding EA_G % Enhancement
2.5	19.5 ± 10.2 (-)
5	6.4 ± 4.2 (-)
10	17.2 ± 8.1
20	13.5 ± 9.2 (-)
40	7.5 ± 6.1
80	9.4 ± 4.2
160	5.6 ± 2.4

TABLE XVI

The effect of ticarcillin on the binding of EA_G to human peripheral monocytes. The ticarcillin was added with the EA_G to the monocyte monolayers. Each figure represents the mean ± 1 SEM of three experiments. The cells were taken from the same donor on different days.

4.0 SUMMARY

These studies were only preliminary, so that few firm conclusions can be drawn from them. However, they do indicate the necessity for further work on the effect of antibiotics on human monocyte receptor expression.

Tobramycin inhibited the binding of EA_G when added with the erythrocytes to the macrophages. This effect was observed whether results from different individuals were pooled or when monocytes were taken from the same individual on different days. However pre-incubation of the monocytes with tobramycin had no effect on Fc receptors, when results from different volunteers were pooled. This was due to individual variation as in one individual tobramycin treatment resulted in a decrease in EA_G binding, while in two individuals an increase was observed.

Azlocillin enhanced binding of EA_G when added with the erythrocytes to the monolayers, although only one individual was studied. Ticarcillin had no effect on Fc receptor expression, which agrees with the results obtained with this antibiotic on rat alveolar macrophages.

CHAPTER V

THE DISCUSSION

1.0 ALVEOLAR MACROPHAGE, PHAGOCYTOSIS AND RECEPTOR EXPRESSION

1.1. Animals

The obvious choice for studying the effects of alginate and antibiotics on alveolar macrophages in relation to CF would be a human model. However human alveolar macrophages from normal, healthy individuals are extremely difficult to obtain especially in a non-clinical setting. The ethics of unnecessary bronchopulmonary lavage on humans is also questionable. Animal studies were therefore instigated to evaluate the future potential of human studies. The rat was chosen as the animal model because alveolar macrophages are readily obtained from these animals and they are cheap and easily maintained. A few studies on human peripheral blood monocytes, the precursors of alveolar macrophages (Thomas et al., 1976) were carried out in the antibiotic study to indicate any antibiotic effects on the human system.

1.2 Techniques

Lung lavage is a standard procedure for obtaining alveolar macrophages from animals and has been described in detail (Holt, 1979c). Although many workers lavage the lung, while intact in the animal, in this study removal of the lung prior to lavage ensured a two-fold increase in cell retrieval. Pre-warming the medium to 37°C before lavage was also required to obtain optimal numbers of macrophages. Lignocaine has been reported to be an effective aid in obtaining high numbers of alveolar macrophages by lavage, although a period of recovery is required for the cells which appear highly vacuolated

after treatment (Holt, 1979c; Miller and Foster, 1981). As high numbers of cells were obtained without the use of lignocaine, this method was not employed as alveolar macrophage function may alter after long periods in culture. Lignocaine is effective in detaching macrophages from the adhesive forces holding them to the alveolar surface, so that cell populations may differ according to the lavage medium employed. When 50ml of lavage fluid was collected, 10^7-2 x 10^7 viable alveolar macrophages were obtained from one rat lung. It was interesting to note that the second 25ml fraction contained twice the number of macrophages than the first 25ml fraction. Therefore the procedure of collecting 50ml was strictly adhered to, so that differences between experiments, as a result of the selection of particular macrophage populations, were minimised. Considerable heterogeneity has been observed within macrophage populations from the lungs of normal rats with respect to cell size, surface morphology and cytochemistry (Holt et al., 1982; O'Neill et al., 1984). Holt et al., (1982) found that macrophages obtained from early washes contained a predominance of large cells rich in Fc receptors (82%) while preparations from later washes contained mainly small macrophages with low Fc receptor avidity. Therefore when the amount of lavage fluid collected is not regulated, Fc receptor expression may differ between experiments.

Macrophages obtained by lavage may not fully represent the resident alveolar macrophage population, so that results must be treated with caution. The main advantage of this technique is that an almost pure population (96-99%) of functionally active alveolar macrophages may be obtained from a healthy animal.

Macrophages were studied as monolayers; in this system the oxygen

tension resembles that found in the lung more closely than when cells are in suspension (Hearst et al., 1980). In studies that involve bacteria, macrophage monolayers have another advantage over cells in suspension, in the former case macrophages do not round up and bacteria can be easily observed within the cell or bound to the membrane. Washing bacteria from cells in suspension is also extremely difficult and a high level of background bacteria usually ensues.

Cells were cultured in serum free medium as the presence of serum increased Fc receptor avidity (Figure 13) and bacterial binding (J. Stewart, personal communication). The absence of serum may also correlate more closely with the environment in the lung alveoli, where the macrophages lie in contact with the surfactant lining of the alveoli (Hocking and Golde, 1979). Alveolar macrophages readily adhered to the glass coverslips and as only a low percentage were still in suspension after one hour, selection of a highly-adherent population did not appear to occur.

1.3 "Lectin-like" receptors

Even at high concentrations ($10^{10} ml^{-1}$) of P.aeruginosa, 100% of alveolar macrophages did not exhibit bacterial attachment (Figure 2). This may be a reflection of the assay system as there was a limit to the number of bacteria that could be overlaid onto the monolayers, or it may be that a subpopulation of these macrophages do not express "lectin-like" receptors. Similar results were found for the binding of Staph.albus to guinea-pig alveolar macrophages (E.J. Glass, PhD thesis, 1981) and mouse alveolar macrophages (Glass et al., 1981). A plateau of bacterial binding was not reached for P.aeruginosa as

concentrations higher than 10^{10} organisms ml^{-1} resulted in a bacterial background that made counting extremely difficult. However, counts over 10^{10} are unlikely to be reached clinically, so observations at higher concentrations would appear to be irrelevant.

Formalin-killed P.aeruginosa bound to the macrophages more readily than live organisms (Figure 2). This may be a result of membrane alteration by the formalin rendering the bacteria more susceptible to binding. Live P.aeruginosa were therefore used in all future experiments to resemble the in vivo situation.

Staph.albus bound to a greater extent than formalin-killed or live P.aeruginosa. This difference may be a result of the coccal structure and gram positive nature of Staph.albus compared to the gram negative bacillus nature of P.aeruginosa.

Mucoid P.aeruginosa did not bind to alveolar macrophages and bacteria were absent from the background, even at concentrations of 10^{11} organisms ml^{-1}. The alginate produced by these bacteria may prevent the organisms from contacting the macrophages as washed bacteria did exhibit binding (results not shown). The alginate may also prevent the bacteria from "sticking" to the glass coverslips, so that the bacterial background is low. Centrifugation of the plates after addition of mucoid P.aeruginosa to aid macrophage bacterial contact, did not result in binding.

D-glucose and D-galactose inhibited the binding of non-mucoid P.aeruginosa to rat alveolar macrophages indicating that binding at least in part was due to the "lectin-like" receptor. Binding of Staph.albus via this receptor has been demonstrated for a number of cell types (Glass et al., 1981). At the highest sugar concentrations (20mm) studied, 100% inhibition was not observed and a maximum

bacterial binding inhibition of 49% was reached at 10mm D-glucose. Binding of bacteria may take place by other sugar components, when one of them is blocked, although experiments in which more than one sugar was added at the same time to the macrophage monolayers were inconclusive (J. Stewart, personal communication). The bacteria may also be binding to the phagocyte surface by mechanisms that are independent of the recognition of these sugars. Non-specific binding involving hydrophobic charge effects and other membrane biomechanical forces may contribute to the observed adherence (Wilkinson, 1976). P.aeruginosa contain lectins in the cell wall (Glick and Garber, 1983) that may bind to specific sugars on the macrophage membrane in a similar way to that observed for C.parvum (Bagg et al., 1981) and E.coli which binds to a mannose-specific lectin (Ofek et al., 1977). However, when bacteria are incubated with sugars prior to binding, inhibition does not occur (J. Stewart, personal communication).

Bacterial attachment via lectin-like receptors does not necessarily bring about phagocytosis. The receptor may act in a similar way to the complement receptor which binds bacteria to the surface so that other receptors may initiate phagocytosis (Fearon, 1984). P.aeruginosa were phagocytosed by mouse peritoneal macrophages when warmed to 37°C after binding at 4°C (Oliver and Weir, 1983). However, unopsonised P.aeruginosa were not phagocytosed by rat alveolar macrophages after binding at 4°C, although Staph.albus were taken up by these cells. This may be due to an insufficient number of bacteria binding to the macrophage, for the initiation of ligand-receptor interactions and subsequent phagocytosis. Alternatively the bacteria may have toxic

effects on the macrophage, so that phagocytosis is inhibited, as P.aeruginosa produce leucocidins. However as these macrophages phagocytose opsonised bacteria and mouse peritoneal macrophages phagocytose unopsonised bacteria this seems unlikely. "Lectin-like" receptors may be important in antigen presentation, because of the possible association with IA (Stewart et al., 1982). Therefore if P.aeruginosa are able to escape initial phagocytosis and antigen presentation, this may enhance their pathogenicity and confer P.aeruginosa with advantages over other bacterial species such as Staph.albus that are phagocytosed unopsonised. However failure to phagocytose unopsonised P.aeruginosa may be a characteristic of the rat alveolar macrophage as remarkable differences in alveolar macrophage receptor expression and function have been observed between animal species (Nguyen et al., 1982).

1.4 Phagocytosis

For P.aeruginosa to be phagocytosed by rat alveolar macrophages, it was necessary to opsonise the bacteria with specific immune serum. Bacteria opsonised with fresh rat serum were not phagocytosed by these cells. P.aeruginosa strains do vary in their requirements for opsonisation (Peterson, 1982). However these bacteria have been found to require opsonisation with specific immune antibodies, before phagocytosis will take place (Reynolds et al., 1975b). Some forms of P.aeruginosa are not opsonised by IgG alone and require an intact complement pathway for opsonisation (Baltimore and Shedd, 1983). The opsonin requirement of a particular bacterial strain may influence its pathogenicity as bacteria that are readily taken up by macrophages will be cleared more effectively than bacteria that are only phagocytosed once an immune response has been initiated. The requirements for

opsonisation may also depend on cell type as mouse peritoneal macrophages phagocytosed P.aeruginosa opsonised with normal mouse serum, while rat alveolar macrophages did not (personal observation).

Agglutination tests on the immune sera raised against P.aeruginosa 492a Rev1 revealed low titres of agglutinating antibody indicating that the bacteria did not bind sufficient antigen for agglutination to occur above dilutions of one in four.

Mucoid bacteria were not studied in the phagocytosis assay as the washing procedure involved in opsonisation renders the bacteria no longer mucoid. It has been shown that alginate blocks the antigenic receptor of the bacteria so that opsonisation of mucoid bacteria does not occur (Baltimore and Mitchell, 1980). Although the alginate has been shown to be antigenic (Bryant et al., 1983; Pier et al., 1983) serum against these bacteria was not raised. However the washing of bacteria opsonised with such serum would still remove the alginate coated with antibody.

A microscopic assay was employed to detect phagocytosis and unphagocytosed bacteria were effectively washed away. Bound bacteria did not remain after the washing procedure as monolayers left at room temperature bound opsonised bacteria but phagocytosis did not occur. When these plates were vigorously washed the bound bacteria no longer remained. Thus the microscopic technique was adequate for the purpose of these studies and phagocytosed bacteria within cell vacuoles were easily distinguished. Trypsin treatment of the macrophages to remove bound bacteria from the cells was not necessary in this system and was disadvantageous as the enzyme appeared to be slightly toxic to the alveolar macrophages.

The optimum conditions for phagocytosis largely depended on the dilution of antibody and concentration of bacteria in the opsonisation mixture. Bacteria that were opsonised and subsequently diluted before addition to the macrophages were not adequately phagocytosed. At high concentrations of bacteria in the opsonisation mixture, phagocytosis was decreased, possibly as a result of ineffective coating of the excess number of bacteria by antibody.

1.5 Fc receptor expression

Receptors for IgG on the macrophage membrane were detected and studied using the rosette technique. This method is well established and involves the sensitisation of erythrocytes with specific antibody that will subsequently bind to the Fc receptors on the macrophage membrane. The antibody used in this study was a monoclonal IgG2b raised in mice against sheep erythrocytes. This particular monoclonal was readily available and gave consistent results. At the time of this study a rat antibody against sheep erythrocytes was not available. Although the mouse monoclonal may not bind specifically to the rat IgG2b receptor, it does bind to IgG Fc receptors on rat macrophages (Boltz-Nitulescu et al., 1981). In the human system, IgG3 binds to a greater extent to Fc receptors than other subclasses of IgG (Naegel et al., 1984). However the mouse monoclonal IgG2b bound to human monocyte Fc receptors and was used to detect these receptors in this study. The drawback of this heterologous system is that although the sensitised sheep erythrocytes form rosettes with alveolar macrophages and monocytes, the specificity of the binding receptor is unknown.

The Fc receptor assay was performed at room temperature (22°C)

to minimise phagocytosis of the EA_G by the macrophages. At 37°C the erythrocytes were readily taken up by the cells (Plate V).

At the highest concentration of antibody (1:2 dilution) 100% positive Fc rosetting cells were not observed (Figure 14). The formation of macrophage-EA_G rosettes occurs only when there are ligand-receptor interactions between many macrophage Fc receptors and the erythrocyte antibody. This union is dependent on the density of bound antibody on the red cells and also on the ease with which the macrophage Fc receptors can move in the plane of the membrane. This failure to attain 100% rosetting cells is not a result of the restriction in the amount of antibody that can be bound to the sheep erythrocytes as even at a dilution of one in two the antibody is subagglutinating. Ox erythrocytes are able to bind more molecules of antibody per cell than sheep erythrocytes (Hallberg et al., 1973). However 100% rosetting cells did not occur, when ox erythrocytes and rabbit antiox antisera were used unless the macrophages were cultured in foetal calf serum (Figure 13). Therefore the low number of rosetting cells may be a result of the size of the alveolar macrophage and the spatial arrangement of the Fc receptors on the ruffled plasma membrane. When the cells are incubated with foetal calf serum their appearance becomes more rounded, i.e. they are less spread out, and Fc receptor avidity increases (Figure 13). The observation that the Fc receptor avidity and size of the smaller more rounded human monocyte do not alter when the cells are incubated with foetal calf serum, supports this theory. Moreover the mouse monoclonal antibody may have a low affinity for rat Fc receptors.

Alternatively there may be a subpopulation of alveolar macrophages

present in the monolayer with low Fc receptor avidity, these may include redundant macrophages or newly recruited monocytes. As previously noted, different lavage fractions contain macrophages with low or high Fc receptor avidity (Holt et al., 1982) and it is well established that subpopulations of macrophages express differences in Fc receptor avidity (Moore and McBride, 1980). In this study heterogeneity in terms of alveolar macrophage cell size was observed and the presence of low avidity Fc receptor bearing cells may partly account for the low number of rosetting cells.

The dilution of sensitising antibody plotted against the percentage of rosette forming cells produces a sigmoidal curve (Figure 14), similar to those reported by Rhodes (1975) for guinea-pig alveolar macrophages, indicating a normal distribution of cellular avidities for IgG amongst these populations.

1.6 Complement receptors

Experiments using the rosette technique with complement coated sheep erythrocytes (described in Materials and Methods) failed to demonstrate the presence of complement (C3) receptors on rat alveolar macrophages. Sheep erythrocytes were sensitised with a monoclonal IgM before the addition of complement components and zymosan depleted serum. The zymosan removes the terminal complement components and therefore allows more C3 to be deposited on the erythrocytes. These results agree with those of Coonrad and Rehm (1982) who failed to detect receptors for CR1, CR2 or CR3 on rat Sprague-Dawley alveolar macrophages. Alveolar complement receptors were detected on peritoneal and alveolar macrophages from Lewis-Wistar rats. Long-Evans and

Wistar rats also lacked C3 receptors on their alveolar macrophages indicating that this phenomena is strain specific. The reason for the lack is unknown but may be related to lipids in the alveolar lining material (Coonrad and Yoneda, 1983). Lavage fluid added to peritoneal macrophages caused the loss of C and Fc receptors by the macrophages.

As the alveolar macrophages in this study lacked C3 receptors, the effect of alginate and antibiotics on their expression was not studied.

2.0 THE EFFECT OF ALGINATE ON ALVEOLAR MACROPHAGES

2.1 "Lectin-like" receptors

Rat alveolar macrophages were shown to possess "lectin-like" receptors (Figure 3), although they bound fewer bacteria than mouse peritoneal macrophages (Oliver and Weir, 1983). Rat alveolar macrophages have a low percentage of Ia bearing cells, approximately 10% (personal observation) and as there appears to be an association between Ia and lectin-like receptors (Stewart et al., 1982) this may account for the low level of bacterial binding to these cells. The alginate used in these studies was produced by a mucoid strain of P.aeruginosa that was originally isolated from a patient with CF. Bacteria were grown under optimum conditions for alginate production and the procedure for extraction was adapted from the method described by Govan (1975). The alginate was free from any contaminating protein as measured on a spectrophotometer and was considered pure enough for the inhibition assays. The non-mucoid revertant of the original mucoid P.aeruginosa was studied as this bacteria is genetically similar, but does not produce alginate or mutate back to the mucoid form (J.W. Govan, personal communication). These two strains thus formed a good system for studying the effect of alginate on bacterial binding.

The alginate inhibited the binding of unopsonised non-mucoid P.aeruginosa to rat alveolar macrophages in a dose-dependent fashion (Figure 4). This inhibition occurred when the macrophages were pre-incubated with alginate and when alginate was added with the bacteria to the monolayers. In the latter procedure, the bacteria were, in effect, made mucoid by addition of the alginate and inhibition was

greater in this case. This is probably due to the alginate acting as an inhibitory matrix on the bacteria and macrophage as opposed to only the macrophage. However, from the results it is uncertain whether the inhibition observed after pre-incubation of the macrophages with alginate is due to a specific lectin-ligand interaction or to the alginate simply forming a mechanical barrier on the macrophage surface. The exopolysaccharide alginate may however be binding to lectin-like receptors in a similar way to other sugars (Weir and Ogmundsdöttir, 1980) and inhibit the binding of organisms other than P.aeruginosa. In this study the binding of Staph.albus to macrophage monolayers was blocked after alginate treatment of the macrophages. In fact Staph.albus binding was inhibited to a greater degree than that of P.aeruginosa (Figure 4). This observation could be due to differences between the bacterial strains and their binding capabilities. Staph.albus binds more readily, but inhibition of binding is greater than with the gram negative P.aeruginosa bacillus. It is also possible that P.aeruginosa may bind to the alginate on the macrophages as it was observed that bacteria sometimes bound to the alginate, which at high concentrations was stuck to the coverslips. These findings do not exclude the possibility that the alginate may coat the macrophage by physiochemical mechanisms, for example hydrophobic interaction rather than binding via a specific receptor. Alginate gels in the presence of bronchial electrolytes so that in vivo it tends to be a viscid fluid rather than free-flowing (Govan, 1983; Govan et al., 1983). Therefore in vivo it is even more likely to act as a physical barrier to prevent bacterial attachment to the phagocytes.

2.2 Phagocytosis

The process of phagocytosis has two phases, attachment of the particle to the cell membrane followed by ingestion, after which intracellular killing usually takes place. In this study phagocytosis was measured by estimating the number of macrophages that had ingested bacteria.

These studies show that phagocytosis of non-mucoid P.aeruginosa is inhibited when monolayers of alveolar macrophages are incubated for 30 minutes with alginate, prior to the addition of the bacteria (Figure 7). Inhibition was increased when the alginate was added with the bacteria and decreased when the cells were vigorously washed to remove the excess alginate. Inhibition was not specific for P.aeruginosa as the phagocytosis of Staph.albus was also inhibited in a dose-dependent fashion, when the macrophages were pre-incubated with alginate (Figure 8). These results confirm and extend observations by other workers. Schwartzman and Boring (1971) showed that alginate inhibited the phagocytosis of bacteria by rabbit neutrophils when the alginate was added to the bacteria and cell mixture. In this study phagocytosis of P.aeruginosa was inhibited to a greater extent than that of E.coli or S.aureus. However phagocytosis was measured by determining the viable bacterial count in the mixture after cell lysis, so that intracellular killing as opposed to phagocytosis was measured. Other results indicating that alginate inhibits phagocytosis have been reported for guinea-pig and rat alveolar macrophages (Ruhen et al., 1980). A recent study has demonstrated significantly reduced phagocytic uptake of mucoid organisms by human neutrophils when compared to non-mucoid strains

(Meshulam et al., 1984). When mucoid strains were grown to produce less alginate their uptake was increased. However these workers washed the bacteria and used normal human serum as the opsonin source, so that the results may be a reflection of strain differences in opsonin requirements as opposed to an effect of alginate, per se. The results in this thesis suggest that the alginate acts as an inhibitory matrix or barrier to prevent attachment and phagocytosis as vigorous washing which removed the alginate from the cells, resulted in phagocytosis returning to control values.

It is possible that the inhibitory effects were caused by a toxic bacterial product contaminating the alginate. To investigate this possibility alginic acid, a chemically similar compound isolated from Macrocystis Pyrifera (Kelp) was studied for its effect on phagocytosis. This compound inhibited the phagocytosis of P.aeruginosa 492a Rev1 when pre-incubated with the macrophages and when added to the monolayers with the bacteria (Figures 9,10). Inhibition was reduced by approximately 50% when the macrophages were vigorously washed after treatment, which agrees with the alginate results. The lower viscosity of this compound enabled the preparation of high concentrations with the result that 100% inhibition was achieved. The observation that low concentrations of commercial alginic acid (0.03mg ml^{-1}) caused inhibition of phagocytosis suggests that the effect is not due to viscosity. The pseudomonal alginate is extremely viscous and solutions above 2mg ml^{-1} were difficult to make whereas alginic acid was not viscous to the eye at 20mg ml^{-1}. Viscosity was not measured due to the lack of equipment. Accurate measurements were not thought necessary in these experiments, as concentrations

were worked out by weight. Therefore *in vivo* where higher concentrations of alginate may occur within the lung, inhibition of phagocytosis may reach higher levels than those observed *in vitro*.

Alginate did not significantly affect cell viability after incubation with the macrophages for 30 minutes, so that the observed inhibitory effects were not a result of alginate toxicity. Viability was slightly decreased at concentrations of 2mg ml^{-1} alginate which may be due to the viscous nature of the compound. The high viscosity may lead to "suffocation" of a small percentage of the macrophages. The percentage of cell death at this concentration was not high enough to be responsible for the antiphagocytic effects of the alginate.

2.3 Opsonisation of bacteria

Opsonisation involves the combination of bacteria or other particles with certain serum proteins, so that the bacteria are more susceptible to phagocytosis (Winkelstein, 1973). Thus any impairment of this process would be advantageous to the infecting bacteria. The alginate from mucoid *P.aeruginosa* was shown to inhibit the opsonisation by specific antibody of the non-mucoid revertant. At high concentrations of alginate, opsonisation was almost totally blocked, as measured by the subsequent inhibition of phagocytosis (Figure 11). This inhibition indicates that the alginate is acting on the bacteria to prevent the attachment of the antibody to the bacterial cell wall. This result does however conflict with the work of Baltimore and Mitchell (1980) who reported that the opsonisation of two non-mucoid strains, which required antibody for opsonisation, was not inhibited by the addition of mucoid material to the opsonophagocytic mixture. However, in this

study, the antiserum, bacteria and neutrophils were incubated together and viable bacterial counts performed to estimate phagocytosis. Therefore the opsoniation procedure was not separated from the phagocytic process in this case. These workers also used a different technique for alginate separation and the material was a very crude extract which may also explain the difference in results. These workers also reported that the immunodeterminant for opsonic antibody in non-mucoid strains is blocked in the mucoid strain as higher concentrations of antiserum were required to achieve the same reduction in mucoid strains (that is intracellular killing) as compared with non-mucoid transformants of the same strain.

In the study reported here, the alginate also appears to be blocking the binding of antibody to a specific immunodeterminant on the non-mucoid bacterial cell wall. The alginate may simply surround the bacteria to prevent antibody attachment. Even if the alginate is washed away from the bacteria during the washing procedure, opsonisation will not have taken place, so that subsequent phagocytosis will not occur. However *in vivo*, the alginate itself would be the outer coat of the bacteria and antibodies against antigenic determinants of the alginate may be produced. Although the evidence suggests that anti-alginate antibody is an ineffective opsonin (Bryant *et al.*, 1983) and that such antibody may even be harmful if it forms immune complexes in CF sera (Berdischewsky *et al.*, 1980; Moss and Hsu, 1982). Anti-alginate serum was not raised in this study because of the problems involved in setting up the assay. For example even if anti-mucoid antibody was produced, opsonisation of the mucoid bacteria would involve a washing procedure that would render the bacteria no longer

mucoid. Schwarzmann and Boring (1971) reported that mucoid P.aeruginosa were phagocytosed by rabbit neutrophils in the presence of serum but the bacteria had been washed prior to the phagocytic assay. Although in vivo, mucoid bacteria may be opsonised, the results shown here on the effect of alginate on phagocytosis indicate that the bacteria may still resist phagocytosis. Also as alginate can inhibit opsonisation of non-mucoid isogenic strains in vitro, in vivo inhibition of the opsonisation of non-mucoid strains may occur, which may result in the maintenance of a non-mucoid population to act as a resevoir from which further mucoid variants could arise. Rough and mucoid strains isolated from CF patients are both coated with antibodies of IgA, IgG and IgM classes (Hann and Holschaw, 1976), so that opsonisation does occur in vivo. The opsonisation of Staph.albus was also inhibited, although to a lesser extent than P.aeruginosa, by the presence of alginate in the serum and bacteria mixture. This low level of inhibition may be a result of unopsonised Staph.albus being taken up by the alveolar macrophages (as previously noted) so that phagocytosis is observed even when opsonisation is totally blocked.

2.4 Fc receptors and the phagocytosis of latex.

Further evidence for the barrier effect of the alginate came from the studies on the binding of antibody sensitized sheep erythrocytes to Fc receptors and the phagocytosis of latex particles. To date, there are no reports in the literature on the effect of alginate on these processes. Both binding of EA_G and the phagocytosis of latex were inhibited when the macrophages were incubated with alginate for 30 minutes prior to the addition of the erythrocytes or latex

particles. The phagocytosis of latex by macrophages is a non-specific process which does not involve specific receptor-ligand interactions (Ryter and DeChastellier, 1982). These results support the hypothesis that the alginate acts as an inhibitory matrix and barrier to prevent the attachment of bacteria and other particles by specific receptors and non-specifically to the macrophage. Inhibition was greater when the alginate was added to the macrophage monolayers with the EA_G or latex particles. This may be a result of the test particles being inhibited from coming into contact with the macrophages by the alginate matrix. During the Fc receptor assay, the erythrocytes were centrifuged gently onto the macrophage monolayers, so that in this case the alginate may prevent adequate settling of the erythrocytes onto the macrophage monolayers.

A study with sheep alveolar macrophages has shown that protein synthesis and latex particle endocytosis is inhibited in the presence of diluted airway mucus (Woodside et al., 1983). The hypersecretion of mucus that is observed in the CF lung may therefore exacerbate any of the inhibitory effects of the alginate.

2.5 Mechanism of action

The alginate from mucoid P.aeruginosa thus appears to have a mechanism of action that is likely to protect the bacteria from the host's immune response by preventing phagocytosis and the initiation of the immune response. This is achieved by the alginate acting as a barrier to prevent the attachment and phagocytosis of bacteria and other particles to the alveolar macrophage membrane, and the opsonisation of non-mucoid P.aeruginosa or other bacteria, by specific

antisera. Other protective mechanisms have also been described for this material, as the presence of alginate has been shown to increase the resistance of P.aeruginosa to surfactants (Govan, 1975) or carbenicillin (Govan and Fyfe, 1978).

It is not unusual for bacteria to secrete material that affords them protection against the hosts immune response. The streptococcal hyalaronic acid capsule has been found to prevent the attachment of two groups of Group A streptococci to mouse peritoneal macrophages (Whitnack et al., 1981). The cell wall material from Cryptococcus neoformans has also been shown to inhibit the attachment and phagocytosis of nonencapsulated yeast cells by macrophages (Kozel and Mastroianni, 1976). This effect is a result of the capsule presenting a surface that is not recognised by the phagocyte. The inhibition can be corrected by opsonising antibody that is specific for any surface determinant on the capsule, that will than bind to the Fc receptor on the macrophage. However unlike alginate which is not a capsule, the Cryptococcus neoformans capsule will only inhibit phagocytosis when it is bound to the yeast and has no direct effect on the macrophage (Kozel and Gotschlich, 1982).

The capsule of S.aureus has been shown to hinder opsonisation by masking cell wall peptidoglycan and therefore interferring with natural immunity (Wilkinson et al., 1979). The M protein of Group A streptococci may act in a similar way. Encapsulated E.coli are more pathogenic than unencapsulated forms as the capsule blocks complement fixation and opsonisation (Horwitz and Silverstein, 1980). Therefore capsular antigens may mask common outer membrane components. Although alginate is non-capsular it may act in an analagous way. In fact as it is not tightly bound to the bacteria it is free to form as an

inhibitory matrix and barrier on other particles and cells, which may in fact account for the enhanced pathogenicity of the mucoid organism.

If the *in vitro* inhibitory effects of alginate on phagocytosis and opsonisation reported in this thesis, occur *in vivo*, they may partly explain the predominance of mucoid *P.aeruginosa* in CF. These effects are also likely to maintain and exacerbate chronic infection with *P.aeruginosa* by protecting the bacteria from the hosts immune response.

3.0 THE EFFECT OF ANTIBIOTICS ON RAT ALVEOLAR MACROPHAGES

Three antibiotics used in the treatment of pulmonary infection due to P.aeruginosa, in CF patients were used in this study. These were tobramycin, an aminoglycoside which is highly active against P.aeruginosa and the B.lactams, azlocillin and ticarcillin. The MIC for each antibiotic against P.aeruginosa 492a Rev1 was estimated to give an indication of the doses required for the study. The MIC of any antibiotic will vary considerably, depending on the bacterial strain and the conditions of the assay. However as P.aeruginosa 492a Rev1 was used in the phagocytic assays, this organism was used to determine the MIC of each antibiotic. Doses below and above the MIC were studied to give a good range. Antibiotic concentrations below the MIC occur in the bronchial secretions of CF patients (Malmborg et al., 1981). The MIC of an aminoglycoside is known to increase in the presence of calcium ions (D'Amato et al., 1975). For azlocillin and ticarcillin calcium ions do not generally influence MIC values, but may for certain isolates i.e. the effect is strain dependent. Therefore 2.18×10^{-3} M Ca^{++} as $CaCl_2 . 2H_2O$ was added to the isosensitest broth to allow for the possible increase in the MIC of the three antibiotics, in the presence of elevated calcium levels that occur in CF patients (Rabin et al., 1980). Alginate has been shown not to bind antibiotics in the presence of physiological saline levels (Tannenbaum et al., 1984) so that it should not affect the MIC.

3.1 Phagocytosis

The results from these studies indicate that at low concentrations certain antibiotics affect alveolar macrophage function in vitro.

Tobramycin and azlocillin inhibit the phagocytosis of opsonised bacteria in a dose-dependent fashion when added to the macrophage monolayers with the bacteria. Only tobramycin had any effect on phagocytosis when the antibiotic was pre-incubated with the macrophage monolayer.

3.1.1 Azlocillin

The inhibitory effects of azlocillin on macrophages appeared to depend on the continual presence of the antibiotic on the macrophage monolayers, as the antbiotic had no effect on phagocytosis except at two concentrations when pre-incubated with the macrophages. Similar requirements have been observed for other antibiotics. For example ampicillin and tetracycline were shown to enhance the phagocytosis of Listeria monocytogenes by human monocytes but only when the opsonised bacteria and antibiotics (at MIC levels) were added together (Adam, 1982). Pre-treatment of the macrophages resulted in the same phagocytic activity as the control. Carbenicillin at 1/16 of the MIC value was similarly shown to increase the phagocytosis of P.aeruginosa by rabbit neutrophils, but only when added to the cells with the bacteria (Nishida et al., 1976). The results reported here also agree with those of Hawkey et al., (1983) who showed that azlocillin and carbenicillin had no effect on the phagocytosis of candida by human neutrophils when the cells were incubated with antibiotic prior to the assay.

The continual presence of the antibiotic in the phagocytosis mixture does not affect the bacteria in the assay system even at high concentrations. Incubation of the opsonised P.aeruginosa

with azlocillin prior to their addition to the macrophage monolayer did not affect the phagocytosis of the bacteria (Table XII). It is therefore unlikely that the bacterial cell wall is rendered more susceptible to phagocytosis by azlocillin in the short time of the phagocytic assay. Azlocillin may bind divalent cations, that are necessary for phagocytic function (Gnarpe and Leslie, 1974). The major effect of tetracyclines on in vitro function of neutrophils has been shown to be mediated by their divalent cation chelating effect (Glette et al., 1984). The results of these studied depended on the concentration of divalent cations in the reaction mixture and differences between antibiotics were shown to be due to their lipid solubility and different cation chelating ability (Glette et al., 1984).

Alternatively azlocillin may bind to the macrophage plasma membrane and alter the receptor avidity for the opsonised particles. Although the observation that azlocillin had no effect or an enhancing effect when pre-incubated with the phagocytes may preclude this possibility. However the azlocillin may only bind loosely to the membrane, so that it is washed away during the washing procedure after pre-incubation. Moreover if azlocillin exerts its inhibitory effects by being loosely bound to the macrophage membrane, its continual presence may be necessary so that high concentrations remain in the medium surrounding the cell. Therefore continual binding of the antibiotic can take place, resulting in a decrease in phagocytosis. Penicillin has been shown to interact with HLA – A and B antigens in such a way that they can no longer be recognised by their specific

antisera (Claas et al., 1983). When lymphocytes from healthy donors were incubated with penicillin and washed before HLA typing, the cytotoxic reactivity of some typing sera with their corresponding antigen was blocked. Penicillin is also capable of binding to polymorphic structures on cell membranes, so that it is feasible that azlocillin may also be capable of binding to specific receptors on the macrophage membrane. Most penicillins are not actively taken up into macrophages (Hand et al., 1983) so that it seems unlikely that during pre-incubation, azlocillin is taken up by the macrophages and rendered inactive.

3.1.2 Ticarcillin

Ticarcillin, although structurally similar to azlocillin (Prince and Neu, 1983) had no effect on phagocytosis with either pre-incubation or addition with the bacteria. Ticarcillin lacks the ureido group that gives azlocillin its potent antimicrobial activity, so that it could be this group that gives azlocillin its anti-phagocytic activity. However ticarcillin can interact with polymorphic non-HLA structures on platelets and neutrophils (Claas et al., 1983), so that it would appear to be capable of binding to cells. Azlocillin also contains less sodium than ticarcillin (McLaughlin et al., 1983) although this would seem to be an unlikely reason for the observed differences between the two B-lactams. Although azlocillin is approximately four times more active than ticarcillin in vitro against P.aeruginosa there is no evidence to suggest that azlocillin is more effective in vivo (Hodson, 1980). If the anti-phagocytic effect of azlocillin occurred in vivo, this may provide an explanation for the incompatability

of *in vitro* and *in vivo* antimicrobial activity.

3.1.3 Tobramycin

Tobramycin, an aminoglycoside, inhibited the phagocytosis of opsonised *P.aeruginosa*, when pre-incubated with the macrophages and when added with the bacteria to the monolayers. Thus this antibiotic may bind to the macrophage membrane, to alter receptor expression, avidity or spatial arrangement so that binding and subsequently phagocytosis is decreased. Binding of an antibiotic to the cell membrane may occur in four ways:

1) insertion into the membrane lipid
2) physical modification of the receptor structure
3) stimulation of the cell to modify receptor function
4) formation of an antibiotic-receptor complex. (Gray *et al.*, 1983)

As tobramycin is antagonised by phosphate, it is possible that the antibiotic bindsto phospholipids via phosphate in the macrophage membrane to exert its action. Rifampicin has been shown to compete with radiolabelled FMLP chemoattractant for binding sites on neutrophils and to displace already bound FMLP, while having no effect on C5a (Gray *et al.*, 1983).

As the bacteria were opsonised with IgG, binding occurred via Fc receptors, so that phagocytosis would take place by the "zipper" mechanism of Griffin *et al.*, (1975). Tobramycin may decrease the number of ligand receptor interactions or cause them to be discontinuous, so that phagocytosis is decreased. As tobramycin decreases phagocytosis after pre-incubation with the macrophages it would appear to be tightly bound to the membrane so that it is not washed away, as may occur with

azlocillin. The observation that phagocytosis is still inhibited after vigorous washing of the treated macrophages, argues in favour of this hypothesis. In contrast a study on human neutrophils showed that when tobramycin-treated neutrophils were washed prior to measuring their chemotactic response inhibition was no longer observed, indicating that in this case the antibiotic was loosely bound and no longer capable of affecting chemotaxis (Seklecki et al., 1978). Tobramycin did not affect the chemotactic factor and was shown to be acting directly on the neutrophil membrane. Tobramycin may therefore bind more tightly to alveolar macrophages than neutrophils or more antibiotic may be required to be continually bound for an effect on chemotaxis to be observed.

Tobramycin is taken up slowly by macrophages (Johnson et al., 1980a) so that the inhibitory effect may be due to stimulation of the macrophage by the antibiotic to result in modified receptor expression and phagocytosis. Antibiotics that have an inhibitory effect on cells often have inhibitory effects on the transcription or protein synthesis of the bacteria (Forsgren and Schmelling, 1977). Tobramycin's rapid bacterial activity is a result of its ability to block bacterial protein synthesis, via the 30S ribosomal subunit. Therefore protein production necessary for the phagocytic response of the macrophage to occur, could be blocked at the transcription stage or later, so that phagocytosis is inhibited. However whether these effects would be exerted during the short incubation times involved in the assays is debatable.

Tobramycin was shown not to have an effect on the phagocytosis of P.aeruginosa after the bacteria had been incubated for 30 minutes with

the antibiotic, so that it would appear to be having a direct effect on the macrophage. The observed decrease in phagocytosis does not agree with the findings of Cifarelli et al., (1982) who showed that tobramycin increased the phagocytic capacity of human macrophages. This discrepancy may be due to the fact that these workers studied cultured human monocytes, which were incubated with the antibiotic for three or 24 hours at only one concentration (2.7μgm ml^{-1}) of tobramycin. Also phagocytosis was measured by the uptake of zymosan.

A time course for the effect of tobramycin was performed (Figure 21) and showed that the inhibition of phagocytosis was highest when the macrophages were incubated with the antibiotic for 15 minutes. By 120 minutes inhibition was no longer significant and had returned to control levels. The loss of effect at two hours may be a result of the antibiotic losing its activity and its uptake and degradation by the macrophages, so that the amount of active tobramycin present is too low to affect phagocytosis. This result would argue in favour of the antibiotic exerting its action on the membrane. The half-life of tobramycin is one and a half to three hours, so that at two hours (two and a half by the end of the assay) some activity may be lost. The question of how tobramycin exerts its effects on the macrophage membrane is dicussed further in the section on Fc receptors.

3.2 "Lectin-like" receptors

Tobramycin inhibited the binding of unopsonised P.aeruginosa to rat alveolar macrophages when the cells were pre-incubated with the antibiotic for 30 minutes. Thus the effect on binding is similar

to that observed for phagocytosis with this antibiotic. The maximal response however occurred after 60 minutes incubation as opposed to 15 minutes for phagocytosis although the inhibition was diminished at 120 minutes. These time differences between binding and phagocytosis may be a result of the temperature at which the assays were carried out. Binding was performed at 4°C, so that the antibiotic would not be taken up during the assay period, whereas the phagocytic process occurs at 37°C so that in this case the antibiotic may be taken up and inactivated by the macrophages. The effect of temperature on phagocytosis was not studied as this process only occurs at 37°C. Phagocytosis is not observed at 4°C, when only binding will occur.

3.3 Fc receptors

3.3.1 Azlocillin

Azlocillin had an inhibitory effect on Fc receptor expression, as measured by the rosette technique, similar to that observed for phagocytosis. Thus inhibition occurred when the antibiotic was added to the monolayer with the erythrocytes, but not when the macrophages were pre-incubated with azlocillin. As phagocytosis of the bacteria opsonised with IgG is initiated by the Fc receptor, this study shows that azlocillin affects the Fc receptor resulting in a decrease in phagocytosis. Azlocillin had no direct effect on the sensitised erythrocytes (Table XII). These results therefore suggest that azlocillin exerts a direct effect on the macrophage membrane to alter Fc receptor expression resulting in a concomitant decrease in phagocytosis. Thus it seems unlikely that the antibiotic has a

direct effect on the process of phagocytosis itself. Although the continual presence of azlocillin is required for an inhibitory effect, this may reflect the *in vivo* situation. *In vivo* it is likely that the alveolar macrophages will continually be in the presence of the antibiotic and bacteria, although the concentration of the former may fluctuate.

3.3.2 Ticarcillin

Similar to the results obtained for phagocytosis, this antibiotic had no effect on Fc receptor expression. The reasons for the observed lack of effect compared with azlocillin have already been discussed.

3.3.3 Tobramycin

When tobramycin was added with EA_G to the macrophage monolayers, Fc receptor expression was inhibited, but when the macrophages were pre-treated for 30 minutes with the antibiotic, there was an apparent increase in Fc receptor expression (Figures 23,24). This latter result seemed paradoxical as the phagocytosis of opsonised bacteria was decreased after 30 minutes pre-incubation. Further investigation revealed that the phagocytosis of EA_G was inhibited in a similar way to that of opsonised *P.aeruginosa*, when the macrophages had been pre-incubated with tobramycin for 30 minutes. It therefore appeared that the antibiotic exerted a different effect on the macrophage receptor than on phagocytic function, but only after pre-incubation. Tobramycin treated macrophages did not bind unsensitised erythrocytes (results not shown) so that the effect was not due to non-specific

binding of erythrocytes. A time course study revealed that the observed Fc receptor enhancement was only transient (Figure 25). Significant enhancement did not occur when the macrophages were treated for 60 minutes and at 120 minutes, inhibition of EA_G binding was observed. Therefore tobramycin caused an initial increase in Fc receptor expression, followed by a decrease. The effect at 120 minutes being similar to that observed for phagocytosis at 30 minutes. Increased macrophage Fc receptor expression, manifest as increased avidity for lightly sensitised erythrocytes is a well documented index of macrophage activation (Rhodes et al., 1981). Increased Fc receptor expression as measured by rosetting techniques has been reported in adjuvant activated as compared to resident alveolar macrophages (Arend and Mannik, 1973). However it is unlikely that activation of the macrophages would occur in 30 minutes as methods for in vitro macrophage activation usually require 24 hour exposure of the cells to the activating agent. Activation would also not account for the subsequent decrease in receptor expression. A new semisynthetic cephalasporin, AC 1370 has been shown to exert its action by binding to mouse peritoneal macrophages to cause their activation (Ohnishi et al., 1984). Soluble factors are then released from the macrophages which in turn activate neutrophils, so that phagocytic activity is increased. However these workers incubated the macrophages for 20 hours with the antibiotic so that activation could occur. The effect of tobramycin over such a long period of time was not investiaged in this thesis as a high percentage of alveolar macrophages do not remain viable after overnight culture in serum free medium. The results of Ohnishi et al., (1984) do

show that antibiotics can affect macrophage function, and to this extent agree with the results reported here.

From Figure 24 it can be seen that the effect of tobramycin on Fc receptors varies considerably according to the temperature at which antibiotic treatment and the Fc receptor assay are carried out. When the macrophages are incubated at 37°C and the assay performed at room temperature, the observed rosette enhancement drops at four times the MIC value. Therefore high tobramycin concentations incubated with macrophages for 30 minutes appear to have a similar effect to that observed with low concentrations (1/2 MIC) incubated with macrophages for 60 minutes.

When the macrophages were incubated at 4°C and the assay performed at 4°C, enhancement was dose-dependent, reaching a maximum at the highest concentration of tobramycin. At 4°C, the antibiotic will remain on the outside of the cell, as phagocytosis or active uptake of molecules will not occur at this temperature (Silverstein et al., 1977). Therefore at 4°C, higher concentrations of antibiotic may be required for a similar effect to that observed at 37°C, when antibiotic uptake will occur. There was also no decline in the response at 4°C indicating that this effect is temperature dependent.

When the incubation and assay were carried out at room temperature enhancement was significantly lower than that observed at 4°C. Similar results were also obtained when the macrophages were treated at 4°C and the assay carried out at room temperature. The possibility that enhancement would increase with higher concentrations of tobramycin at this temperature, was not investigated in this study. It is possible that at room temperature, the antibiotic is more loosely bound to the

membrane, so that enhancement is not as marked as that observed at 4°C.

From these results it would appear that tobramycin may exert its effects in two ways. As enhancement occurs at 4°C, tobramycin does not necessarily depend on active metabolism of the cell. In this case the antibiotic appears to bind to the macrophage membrane, probably forming an antibiotic receptor complex that results in alteration of the receptor and increased binding of EA_G to the cell. A preliminary experiment on the effect of time on this response revealed that enhancement still occurred when the cells were incubated with tobramycin at 4°C for one hour and two hours, although enhancement reached a maximum at one hour. Tobramycin may also exert its action by altering the cell once it has been taken up. This effect may result in decreased phagocytosis or receptor expression. Thus at 30 minutes more antibiotic is bound to the membrane than present inside the cell and receptor expression is enhanced, whereas at 120 minutes, the concentration of antibiotic may be higher within the macrophage and inhibition is observed. Therefore at 4°C when the antibiotic is not taken up, inhibition of EA_G binding is not observed even after two hours.

At room temperature, there may be a slow rate of uptake of the antibiotic, which may only be loosely bound to the membrane and the metabolic rate of the macrophage is decreased, with the overall effect that only slight Fc receptor enhancement is observed at this temperature compared to that at 4°C.

It is also possible that tobramycin may act in a different way at 37°C than at 4°C. At 4°C tobramycin will be acting directly on

the membrane but at 37°C, it may exert its effects from within the macrophage and alter the turnover of the Fc receptor, so that an initial increase is followed by a decrease as intracellular pools are reduced. If tobramycin does have two mechanisms of action, one outside the cell, and one inside the cell, the overall result would be determined by temperature and time, as the results suggest.

After two hours pre-incubation, inhibition occurs at all concentrations studied, while at one hour the response is variable. Maximum inhibition occurs at three hours after which the response levels off. As the half-life of tobramycin is one and a half to three hours, the decline in effect after three hours may be a result of the antibiotic going "off" as well as its degradation and inactivation by the macrophages. The observation that Fc receptor expression is inhibited when tobramycin is added with the erythrocytes indicates that the mechanism of action is different again in this system. It is possible that the red cells bind the antibiotic so that less is available for binding to the membrane. On the other hand the presence of the red cells may alter the binding of the tobramycin, and the arrangement of the receptor so that inhibition rather than enhancement is observed. It is interesting to note that the observed inhibition reaches a maximum at MIC, thereafter declining to insignificant levels.

Although the sensitised sheep erythrocytes become more susceptible to phagocytosis after tobramycin treatment, they do not bind more readily to the macrophage. This effect seems paradoxical but may be a result of the antibiotic altering the erythrocyte membrane in such a way that bound cells are more readily phagocytosed. Therefore a higher proportion of bound untreated erythrocytes remain unphagocytosed.

At the present time there are no reports in the literature to confirm or disagree with these findings that tobramycin has a direct effect on a specific receptor.

3.4 Studies on supernatants from tobramycin treated macrophages

As tobramycin treatment of alveolar macrophages resulted in an increase in Fc receptor expression, followed by a decrease, it was postulated that Fc receptors could be released into the supernatant during incubation with the antibiotic. This hypothesis was investigated by analysing the supernatants from tobramcyin treated macrophages.

The binding of EA_G to macrophages was found to be inhibited after the erythrocytes had been pre-incubated with supernatnants from tobramycin treated macrophages. On incubation with the macrophages, the supernatants enhanced the binding of EA_G. Therefore it seemed likely that Fc receptor (Fc-R) material was present in the supernatants and was binding to the antibody on the EA_G to inhibit their binding to macrophage Fc receptors and attaching to the macrophage membrane to enhance the binding of EA_G. Other receptors, including the lectin-like receptor, present in cell lysates can be inserted into the macrophage membrane (J. Stewart, unpublished results). Fc-R like material has been isolated from supernatants of peripheral human mononuclear cells, in consequence of a $4^{\circ}C$-$37^{\circ}C$ temperature shift (Sandor et al., 1978). Incubation of this material with sensitised erythrocytes, resulted in inhibition of their binding to monocytes. Other workers have tested supernatants from normal or activated lymphocytes for the

presence of Fc-R-like material (Caraux and Serrou, 1979). They found a soluble factor that combined with Fc fragment antigen complexed IgG to inhibit the binding of immune complexes to Fc receptors on cell surfaces. This soluble factor also caused the haemagglutination of erythrocytes sensitised with a subagglutinating dose of IgG.

Supernatants from untreated macrophages also contained some Fc-R like material, so it would appear that tobramycin increases the "normal" release of the Fc receptor from the membrane. This phenomena would explain the decrease in enhancement observed at the 60 minute and 120 minute incubation times of the macrophages with the antibiotic. However it does not explain the initial enhancement observed after the 15 minute and 30 minute tobramycin incubation times. The initial action of tobramycin may be to increase the number of Fc receptors or their spatial arrangement in ways that were previously discussed, with a resulting increase in the number of Fc receptors that can be shed into the medium. There may be two populations of Fc receptors present on the macrophage membrane, one that is shed and one that remains firmly bound (Sandor, et al., 1979).

The number of shed receptors was dose-dependent but did not significantly increase after two hour treatment of the macrophages (Figures 28,29). This may indicate that lost receptors are not replenished, so that shedding effectively decreases the number on the membrane or forms an equilibrium. Sandor et al., (1979) reported that in their system a continuous secretion of the Fc-R material did not occur. Lost receptors that are not replenished, would explain the inhibition observed at two to five hours of tobramycin treatment of macrophages. Supernatants taken from macrophages treated for 30

minutes appeared to contain little Fc-R material as inhibition of binding of treated EA_G did not occur. Thus initial Fc receptor enhancement would appear to be followed by an increase in the shedding of the receptors from the membrane.

Further evidence for the presence of Fc receptors in the supernatant came from haemagglutination studies. Supernatants were incubated for one hour with formalised erythrocytes sensitised with the monoclonal IgG2b antibody ($F.EA_G$) to avoid lysis. The erythrocytes were washed and the resulting supernatant concentrated by freeze-drying. This supernatant, unlike unabsorbed supernatants did not cause the agglutination of EA_G. Therefore the material responsible for agglutination had been removed by the $F.EA_G$ further indicating its Fc-R nature.

3.4.2 Polyacryamide gel electrophoresis

Page gel analysis revealed that more material was present in two bands at approximately 47,000 and 60,000 daltons in supernatants from tobramycin-treated macrophages than control supernatants. Some cellular material is also present in the supernatants of untreated control cells, but there seems to be a general increase in the release of material from tobramycin treated macrophages. This observation was not thought to be a loading effect as 100µl of each sample was precisely measured into each chamber and the experiment was repeated (Plate VII). The quantity of material in the band corresponding to 47,000 daltons was almost double in the supernatant from treated macrophages to that of supernatant from untreated cells. The exact molecular weight of the rat alveolar macrophage Fc receptor is unknown but the molecular weights of other Fc receptors range from

35,000 to 100,000 daltons. Two polypeptide chains with molecular weights of approximately 60,000 and 47,000 daltons have been isolated from the Fc receptor of a mouse macrophage cell line (Melman and Unkeless, 1980). It is possible that these two bands represent the Fc receptor that from the studies described earlier appears to be shed from the macrophage membrane.

Supernatants that have not been dialysed and still contain tobramycin appear to affect the erythrocyte membrane to a greater extent than control supernatants (Plate VII). All the bands are increased in supernatants from tobramycin treated cells indicating that greater lysis has occurred. Tobramycin would appear to be responsible for this effect as dialysed supernatants did not cause increased lysis or band formation (results not shown). This effect would explain the increased phagocytosis of EA_G after tobramycin treatment as it indicates alteration or rupture of the membrane that may facilitate phagocytosis but not binding of the EA_G.

4.0 EFFECT OF ANTIBIOTICS ON HUMAN MONOCYTES

Initial studies on human peripheral blood monocytes indicated that tobramycin and azlocillin had an effect on the Fc receptor expression of these cells, while ticarcillin had no effect. This follows the pattern observed for rat alveolar macrophages.

Tobramycin inhibited the binding of EA_G when the antibiotic was added to the monolayers with the erythrocytes. Inhibition was significant when results from different individuals were pooled and when one individual was studied at different times (Figure 31). However when the monocytes were pre-incubated with tobramycin, individual variation affected the results. When results from different individuals were pooled, the antibiotic appeared to have no effect. On closer investigation, however it was observed that tobramycin inhibited or enhanced Fc receptor expression depending on the individual under study. Overall these opposite effects would cancel out and tobramycin would appear to have no effect on Fc receptors. Individual variation is not uncommon for human macrophage function. For example alveolar macrophages from different individuals were shown to vary in their antigen presenting capacity (Toews et al., 1984). This longitudinal study demonstrated that macrophages from some individuals present antigen, some suppress lymphocyte proliferation while others exhibit responses that fall between the two. Therefore longitidunal studies may give different results from studies that involve pooling results from different individuals and their use is indicated in this study.

Similar to the study on rat alveolar macrophages, tobramycin appears to have different mechanisms of action depending on the

assay procedure employed. Individual variation may be a result of differences in Fc receptor avidity and affinity. Differences observed between pre-incubation of macrophages with tobramycin and addition of the antibiotic with the erythrocytes to the macrophage monolayer would have to be investigated further at an individual level.

Azlocillin caused enhancement of EA_G binding to monocyte Fc receptors, but the continual presence of the antibiotic was required. In this case the results do not agree with those observed with rat alveolar macrophages. This may be a result of the different nature of the two cell types and would have to be examined more fully.

Although only a few studies on human monocytes were carried out, they yield enough information to indicate that antibiotic effects may occur in the human system and that further work is necessary.

5.0 GENERAL DISCUSSION

Alginate has been shown to inhibit the binding and phagocytosis of P.aeruginosa to alveolar macrophages which may partly explain the chronicity of infection with the mucoid organism. Tobramycin and azlocillin have also been shown to inhibit the phagocytosis of P. aeruginosa, while ticarcillin has no effect. Therefore these two antibiotics would appear to exacerbate the effects of the alginate on macrophage function. P.aeruginosa once established in the CF lung are seldom if ever eradicated despite treatment with these antibiotics alone or in combination. However therapy with these antibiotics has been associated with clinical improvement (Marks et al., 1976) and azlocillin and tobramycin in combination are effective at temporary eradication of the mucoid organism (Møller and Høiby, 1981). The clinical improvement of antibiotic treated patients indicates that the advantageous antimicrobial activity of the antibiotic outweighs any disadvantageous effect it might have against the host cells. However this may not always be the case, for there is a fall in efficacy when antipseudomonal treatment is repeated (Møller and Høiby, 1981). At the beginning of a bacterial infection, eradication may be achieved by antibiotics regardless of their effect on the host cells. However once the infection is established and mucoid strains have emerged, the effect on the host cell may become more important. During chronic infection, that occurs in CF patients, the immune system may become hyperfunctional and actually cause tissue damage. In this case suppression of the immune response by an antibiotic may be advantageous to the host. Thus the benefit to be derived from

an antibiotic may depend on the time and stage of the infection at which it is given. The antimicrobial effect of an antibiotic *in vitro* may not always be reflected *in vivo* even when standard doses give serum or tissue levels exceeding minimal inhibitory concentrations. On the other hand an antibiotic may succeed clinically contrary to expectations (Ernst and Sande, 1981). These discrepancies may be attributable to host factors and the effects of an antibiotic on the patients own immune defence system, which are not taken into account during *in vitro* testing.

The results discussed in this thesis however were all obtained from *in vitro* stuides, done mainly on rat-macrophages so that extrapolation to the human situation *in vivo* is difficult. *In vivo* many factors have to be taken into consideration, for example the immune status of the patients, the extent and time of the infection and previous antimicrobial therapy. However it is arguable that antibiotic effects on the hosts immune response, as indicated in these studies may be relevant to the antibiotic management of CF patients.

6.0 CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDIES

6.1 Alginate studies

Alginate, a mannuronic and guluronic acid polymer produced by mucoid P.aeruginosa acts as a barrier or inhibitory matrix to prevent the attachment and phagocytosis of bacteria and other particles by rat alveolar macrophages. Similarly alginate acts to inhibit the opsonisation of non-mucoid P.aeruginosa by specific antibody. This action of the alginate may confer a selective advantage on the mucoid form of P.aeruginosa and partly explain the pathogenicity of this organism.

It has recently been established that in the presence of bronchial electrolytes, mucoid bacteria can be washed without the loss of cellular alginate (J.G. Govan, personal communication). This should now enable studies on the mucoid form of P.aeruginosa to be carried out. The ability of anti-alginate antibody to act as an effective opsonin could be determined. The ability of alveolar macrophages to bind and phagocytose the opsonised mucoid organism could also be evaluated. Thus the experiments described in this thesis could be extended, and the role of alginate investigated further.

The alginate may surround or coat the macrophage to exert its inhibitory effects. This possibility could be investigated by immunofluorescence. Fluorescein labelled anti-alginate antibody could be prepared and added to macrophages that had been incubated with alginate. Using microscopy, macrophages showing fluorescence would indicate the presence of alginate on the cell membrane. The same technique could be applied to investigate coating of non-mucoid bacteria with the alginate during the opsonisation procedure.

A method for intra-tracheally infecting rats with P.aeruginosa enclosed in agarose beads has been described (Cash et al., 1979). In vivo studies could be carried out using this rat model to indicate the importance of the immune system during the infection and the effector cells that are most important in the eventual eradication of the infection. This work would involve the use of immunosuppressive and activating drugs to determine their effect on the time for eradication of the bacteria. Also differences in infection between mucoid and non-mucoid P.aeruginosa could be established. Alveolar macrophages could be obtained from infected animals and tested for any functional differences from cells of healthy animals. Subpopulations could be separated using an elutriator and differences determined between control and infected alveolar populations, for example their ability to present antigen, phagocytosis and Fc receptor avidity.

Conclusions drawn from animal studies cannot be extrapolated to the human situation. Therefore the need for human studies may arise. Experiments on the effect of alginate on human alveolar macrophages could be carried out, or as these cells are difficult to obtain, human peripheral blood monocytes.

6.2 Antibiotic studies

These studies have shown that low concentrations of antibiotics may affect alveolar macrophage function and receptor expression. Although ticarcillin had no effect on phagocytosis or Fc receptor expression, azlocillin had an inhibitory effect that was dependent on the continual presence of the antibiotic. On the other hand tobramycin inhibited phagocytosis but enhanced Fc receptor expression

on alveolar macrophages that had been incubated with the antibiotic for 30 minutes. This response was found to be dose, time and temperature dependent. Further work on the mechanism of action of tobramycin indicated that this antibiotic caused an initial increase in Fc receptor expression followed by an increased shedding of the receptor from the membrane into the surrounding medium. Initial studies on human peripheral blood monocytes also indicated that azlocillin and tobramycin affected Fc receptor expression of these cells. These results suggest an enormous scope for future work and indicate the necessity of many more experiments to extend and clarify these observations. Future studies that could continue from this work are outlined below:

1) The determination of the quantitative effect of tobramycin on Fc receptors by using a more sensitive assay for their detection. For example radio-labelled antibody aggregates will bind to Fc receptors. Using this technique scatchard plots could be drawn so that the number of receptors/cell and affinity of the receptor could be calculated.

2) Further studies on supernatants from antibiotic-treated macrophages could be carried out. Polyacrylamide gel electrophoresis of EA_G absorbed supernatants could show whether the material absorbed by the sensitised erythrocytes, corresponded to the Fc receptor. The presence of Fc receptors in the supernatant will inhibit the C1 or complement dependent haemolysis of IgG but not IgM sensitised erythrocytes (Fridman et al., 1981). Quantitation of Fc receptor material can be estimated by this test system. One unit of Fc receptor has been defined as the quantity required to inhibit 50%

of hemolysis of EAIgG in excess of complement. The number of units in various supernatants thus represents the reciprocal of the dilution of a supernatant that inhibits 50% hemolysis. Thus the effect of tobramycin on the shedding of Fc receptors could be estimated quantitively. Other assays that could be carried out to confirm the presence of the Fc receptor include the inhibition of binding of radiolabelled C1q, antibody dependent cell cytotoxicity, enhanced precipitation of antigen antibody complexes and the arming of Fc receptor negative cells.

3) Further studies on human monocytes could be performed to extend the reported observations. The effect of pre-treatment of tobramycin on Fc receptor expression in different individuals could be more thoroughly examined. A larger number of individuals could be studied, so that the predominating effect could be determined. Also clarification of the apparent reverse effect of azlocillin on human monocytes as compared to rat alveolar macrophages could be investigated by performing more experiments, and by studying the effect of time, temperature and dose on the results.

4) Other cell populations could be studied. The cells in this thesis do not possess complement receptors, so these receptors were not investigated. However many other populations of cells do possess these receptors and the effect of antibiotics on this receptor could be investigated in a similar way to that described in this thesis for the Fc receptor.

5) The effect of antibiotics on other macrophage functions could be investigated. These would include antigen presentation, intracellular killing and the production of lymphokines and other mediators.

During a hyperimmune response tissue damage may occur as a result of the over-production of various enzymes such as elastase by macrophages and neutrophils. Therefore the effect of an antibiotic on enzyme release may be important in the long term treatment of hyperimmune patients.

6) In vivo studies on animals treated with antibiotics would ascertain whether macrophage function and receptor expression is modified after such treatment. This could be further extended to work on normal healthy volunteers as some studies have already described (Grant et al., 1983; Cullen et al., 1983).

7) This study has concentrated on the mechanism of action of tobramycin on the Fc receptor, however other antibiotics may have similar or apposite effects. A range of other antibiotics that are used for long term therapy could be examined for their effect on macrophage receptors. Comparative studies of different antibiotics could be clinically important and indicate the choice of antibiotic for a particular patient.

The cystic fibrosis patient is only one of many compromised individuals in whom even a marginal influence on host immune response may have a significant effect on the eventual outcome of antibioitc treatment. Ultimately, studies on patients undergoing long term antibiotic therapy could be carried out to evaluate the effect of the drug on the hosts immune system.

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PUBLICATIONS

The following work connected with this thesis has been or is about to be published:

OLIVER, A.M. and WEIR, D.M. (1985) The effect of Pseudomonas alginate on rat alveolar macrophage phagocytosis and bacterial opsonization. Clin. Exp. Immunol., 59: 190-196.

OLIVER, A.M. and WEIR, D.M. The *in vitro* effect of three antibiotics on rat alveolar macrophages. J. Clin. Lab. Immunol., (In press).

Clin. exp. Immunol. (1985) **59,** 190–196.

The effect of *Pseudomonas* alginate on rat alveolar macrophage phagocytosis and bacterial opsonization

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SUMMARY

Alginate obtained from a mucoid strain of *Pseudomonas aeruginosa* was shown to inhibit the phagocytosis of an isogenic non-mucoid revertant by rat alveolar macrophages. Phagocytosis of *Staphylococcus albus*, binding of sensitized sheep erythrocytes to Fc receptors and uptake of latex particles were also inhibited. These results suggest that the alginate acts as a barrier, surrounding the macrophage preventing the attachment of bacteria or other particles to the plasma membrane. This conclusion was supported by showing that alginic acid, a polysaccharide from seaweed structurally similar to alginate also inhibited the phagocytosis of non-mucoid *Ps. aeruginosa*. The alginate also inhibited opsonisation of the non-mucoid revertant by a non-agglutinating hyperimmune serum. It is proposed that alginate confers a selective advantage on mucoid producing forms of *Ps. aeruginosa* by impairing the host immune response by its action on alveolar macrophages and opsonization of bacteria.

Keywords alveolar macrophages cystic fibrosis *Pseudomonas* alginate phagocytosis opsonization

INTRODUCTION

Strains of *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis (CF) have been found in culture to produce alginate, a D-mannuronic and D-guluronic acid polymer linked by *O*-acetyl groups (Doggett *et al.*, 1966). The presence of these mucoid strains in the lungs of CF patients is associated with a poor prognosis (Høiby, Andersen & Bendixen, 1975) and contributes to the persistence of the infection (Doring & Høiby, 1983). This suggests that the alginate produced by the bacteria may be a contributory factor in the pathogenesis of pseudomonas infection in CF.

Failure to eradicate mucoid *Ps. aeruginosa* strains is a major problem in the treatment of CF and is not thought to be due to a general immune defect (di Sant'Agnese & Davis, 1976). However, it is possible that immune function in the lung may be impaired and exacerbated as a result of alginate production by *Ps. aeruginosa*.

Previous work in this laboratory has shown that the alginate obtained from a mucoid producing strain of *Ps. aeruginosa* inhibited the binding of a non-mucoid revertant to mouse peritoneal and pulmonary macrophages (Oliver & Weir, 1983). Schwartzmann & Boring (1971) have reported that alginate can inhibit the phagocytosis of non-mucoid strains by rabbit neutrophils and preliminary studies have been reported on guinea-pig alveolar macrophages (Ruhen, Holt & Papadimitriou, 1980).

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The purpose of this study was to extend these observations and to investigate these effects in more detail as the pathogenic properties of alginate are not understood.

We used alginate separated from a mucoid strain of *Ps. aeruginosa* to determine its effect on the opsonization of a non-mucoid revertant strain and the phagocytosis of opsonized bacteria and other particles by rat alveolar macrophages.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (250 g) were obtained from the Centre for Laboratory Animals, University of Edinburgh.

Bacteria. Ps. aeruginosa strains were obtained from Dr J. R. W. Govan, Bacteriology Department, University of Edinburgh. Strain 492a is a mucoid, alginate producing strain isolated from the sputum of a CF patient and 492a Rev 1 is an isogenic non-mucoid revertant of this strain. The bacteria were grown for 24 h at 37°C on nutrient agar (Columbia agar base, Oxoid) and harvested by scraping from the plate with a glass slide. Bacterial concentrations were calibrated with a spectrophotometer (SP30; Pye Unicam) at 540 nm. A reading of 0·3 was equivalent to 5×10^8 bacteria/ml.

Staphylococcus albus. A strain of *S. albus* was obtained from the departmental teaching collection. The organisms were grown in horse digest broth, harvested in log phase and killed by 24 h exposure to 0·5% formalin at 4°C. Concentrations were calibrated spectrophotometrically as above ($0{\cdot}42 = 2 \times 10^7$ bacteria/ml).

Extraction of alginate. The alginate was extracted from *Ps. aeruginosa* 492a, as previously described (Oliver & Weir, 1983).

Preparation of sensitized sheep erythrocytes (EAG). EAG were prepared as described elsewhere (Glass, Stewart & Weir, 1982). The anti-sheep erythrocyte antibody used was a murine monoclonal (IgG2b–MAS 013c, clone Sp2; Sera-Lab. Ltd., Crawley Down, Sussex, UK).

Preparation of hyperimmune serum. Heat killed *Ps. aeruginosa* 492a Rev 1 were washed in saline adjusted to a final concentration of 5×10^9 bacteria/ml and mixed with an equal volume of Alhydrogel (2% solution) (Miles Laboratories). The mixture was incubated at 37°C for 1 h, washed and resuspended in saline to give a final concentration of 5×10^9 bacteria/ml. Five rats, each received four weekly i.p. injections of 1 ml of bacteria. They were killed and exsanguinated 1 week after the last injection. The serum was collected, pooled and heat inactivated at 56°C for 30 min and stored at −20°C.

Opsonization of bacteria. Hyperimmune serum was diluted 1:80 with Hanks' balanced salt solution (HBSS) containing 0·1% gelatin (GHBSS) and 9 ml was added to 1 ml of a suspension of 5×10^9 bacteria/ml. The mixture was incubated for 20 min in a shaking water bath at 37°C, washed and finally resuspended in HBSS to give a final concentration of 5×10^8 bacteria/ml.

Inhibition of opsonization. Serum was diluted 1:40 with GHBSS and mixed with an equal volume of alginate at different concentrations. Opsonization was then carried out as described above.

Macrophages. Animals were killed with sodium pentobarbitone (50 mg/animal) and exsanguinated. The trachea and lungs were removed and lavaged using a tracheal cannula with Dulbecco's phosphate-buffered saline (DPBS), containing heparin (10 units/ml), pre-warmed to 37°C. Fifty millilitres of lavage fluid was collected and centrifuged at 200*g* for 10 min. The sedimented cells were resuspended in Eagles MEM without serum (buffered with sodium bicarbonate and supplemented with glutamine, final concentration 2 mM) to give a final viable cell count of 2×10^5 macrophages/ml.

Preparation of monolayers. Monolayers were prepared as previously described (Oliver & Weir, 1983). Non-adherent cells were removed by washing with warm Eagles MEM.

Phagocytosis. Macrophage monolayers were overlaid with 1 ml of bacterial suspension in HBSS and incubated for 30 min at 37°C. Unphagocytosed organisms were removed by vigorous washing with HBSS. Coverslips were air dried, fixed in methanol and stained with May–Grunwald–Giemsa. Triplicate coverslips were used and 200 macrophages counted on each coverslip. The results were expressed as the percentage of macrophages that had phagocytosed five or more bacteria.

Fc receptor assay. Two millilitres of EAG ($2{\cdot}5 \times 10^7$ red cells/ml) were added to each monolayer, centrifuged for 4 min at 30*g* and incubated for 30 min at 22°C (room temperature). Non-adherent erythrocytes were removed by gentle washing with HBSS and the coverslips dipped into 1% formal-HBSS to prevent red cell lysis. Triplicate coverslips were used and on each coverslip 200 macrophages were counted. Results were expressed as the percentage of macrophages binding two or more erythrocytes.

Phagocytosis of latex. The macrophage monolayers were overlaid with 1 ml of HBSS and 20 μl of latex suspension (DIFCO Laboratories, Surrey, UK) (adjusted to read 192 at 540 nm on a spectrophotometer) and incubated for 1 h at 37°C. Non-phagocytosed particles were removed by repeated washing with HBSS. Triplicate coverslips were used and on each coverslip 200 macrophages counted. The results were expressed as the percentage of cells that had taken up five or more latex particles.

Inhibition studies. Cell monolayers were incubated with 1 ml of alginate at physiological concentrations (Ohman & Chakrabarty, 1982), diluted in HBSS for 30 min at 37°C. The cells were washed and assays performed as described above. In other assays the alginate was added with the indicator cells to the macrophage monolayers.

Calculations. Percentage inhibition was calculated as the difference between control (C) and test (E) divided by the control × 100:

$$\frac{C-E}{C} \times 100.$$

Statistics. Statistical analysis was performed by the Student's *t*-test.

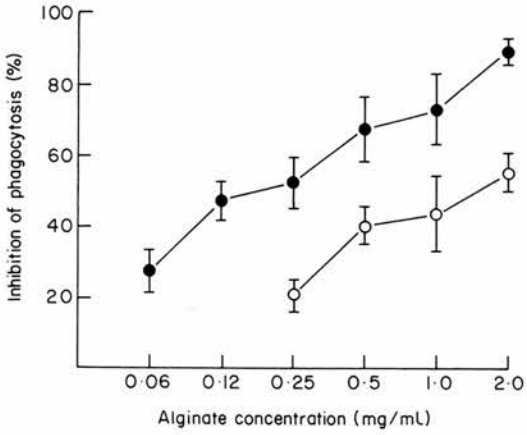

Fig. 1. Inhibition of phagocytosis of non-mucoid *Ps. aeruginosa* by alginate. O——O = pre-treatment of macrophages with alginate; ●——● = addition of alginate with bacteria. Each point represents the mean of four experiments ± 1 s.e.

Table 1. Effect of alginate on the phagocytosis of *S. albus*

	Inhibition of phagocytosis (%) ± 1 s.e.	
Concentration (mg/ml)	Pre-treatment of macrophages with alginate	Addition of alginate with bacteria
0·25	40·5 ± 5	45·6 ± 4·1
0·5	52·1 ± 2·5	54·5 ± 2
1·0	61·3 ± 8·3	72 ± 3·2
2·0	80·5 ± 4·4	87 ± 5·1

Table 2. Effect of alginic acid on phagocytosis of *Ps. aeruginosa* 492a Rev 1

Concentration (mg/ml)	Inhibition of phagocytosis (%) ± 1 s.e.	
	Pre-treatment of macrophages with alginic acid	Addition of alginic acid with bacteria
0·3	29 ± 6	34·8 ± 11
0·6	37 ± 10	44·8 ± 7
1·25	41 ± 11	56·7 ± 6
2·5	63 ± 7	77·3 ± 4
5	78·7 ± 5·2	92·7 ± 0·3
10	86·6 ± 3·3	95·5 ± 4·5
20	100 ± 0	100 ± 0

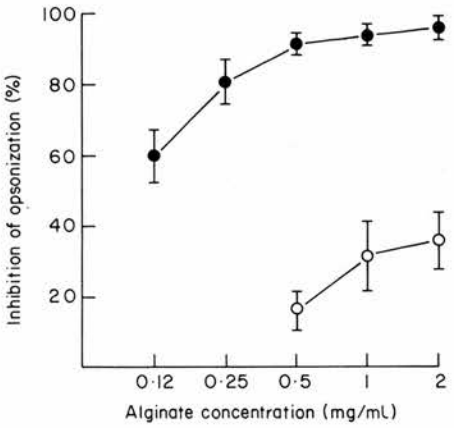

Fig. 2. Inhibition of bacterial opsonisation by alginate. ●——● = non-mucoid *Ps. aeruginosa*; ○——○ = *S. albus*. Each point represents the mean of five experiments (*Ps. aeruginosa*) or three experiments (*S. albus*) ± 1 s.e.

RESULTS

The effect of alginate on phagocytosis

For phagocytosis to occur *Ps. aeruginosa* required to be opsonized with hyperimmune serum, whereas *S. albus* could be opsonised with heat-inactivated normal serum presumably because of the presence of anti-staphylococcal antibody. It is not possible to use mucoid *Ps. aeruginosa* in this assay as the washing procedure after opsonisation causes removal of the alginate, so that the bacteria are no longer mucoid.

Pseudomonas alginate inhibited the phagocytosis ($P < 0{\cdot}05$) of non-mucoid *Ps. aeruginosa* (Fig. 1) and *S. albus* (Table 1) when pre-incubated with the macrophage monolayer for 30 min. This inhibitory effect was reduced by 50% if the macrophages were vigorously washed five times after alginate treatment. The phagocytic capacity of the macrophages returned to normal when these macrophages were re-incubated in Eagles MEM alone for 30 min. Inhibition reached a maximum of 90% when the alginate was added to the macrophage monolayer together with the opsonized bacteria (Fig. 1).

Alginic acid, isolated from *Macroystis pysifera* kelp (Sigma) inhibited the phagocytosis of the non-mucoid revertant in a dose-dependent fashion (Table 2) indicating that the effect of alginate was not due to bacterial derived toxic impurities in the preparation.

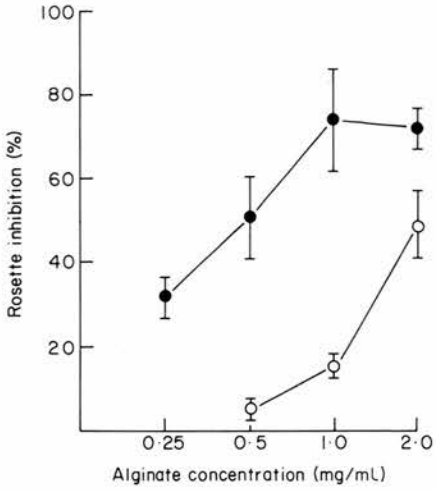

Fig. 3. Inhibition of rosette formation by sensitized sheep erythrocytes by alginate. ○——○ = pre-treatment of macrophages with alginate; ●——● = addition of alginate with sheep erythrocytes. Each point represents the mean of four experiments ± 1 s.e.

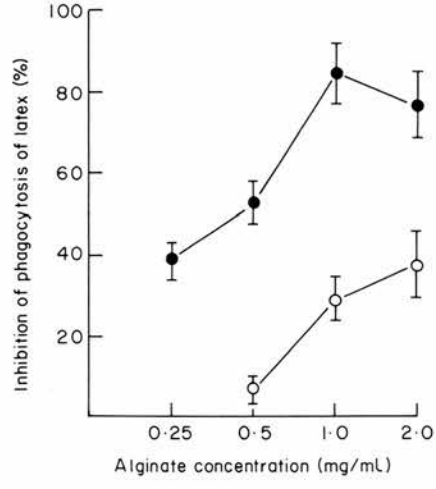

Fig. 4. Inhibition of phagocytosis of latex particles by alginate. ○——○ = pre-treatment of macrophages with alginate; ●——● = addition of alginate with latex. Each point represents the mean of three experiments ± 1 s.e.

Effect of alginate on opsonization

Opsonization of the non-mucoid revertant by hyperimmune serum was inhibited ($P < 0{\cdot}05$) at 0·12 mg/ml and 0·25 mg/ml and almost totally inhibited ($P < 0{\cdot}001$) at concentrations over 0·5 mg/ml (Fig. 2). The inhibitory effect was not due to inadequate pelleting of the bacteria in the alginate and serum mixture during the washing procedure as colony counts showed that there was no difference in the control and experimental bacterial concentrations of the pellets. Inhibition of opsonisation of *S. albus* was less than that observed with *Ps. aeruginosa*. This may be due to the fact that unopsonized *S. albus* are phagocytosed by alveolar macrophages.

Effect of alginate on Fc receptors

The effect of alginate on Fc receptors was studied with an antibody dilution that gave 30–40% binding of sensitized sheep erythrocytes to the alveolar macrophages.

Alginate reduced ($P < 0{\cdot}05$) the binding of sensitized sheep erythrocytes to the macrophages (Fig. 3). This inhibition was greater when the alginate was incubated together with the sheep erythrocytes than when the macrophages were pre-treated for 30 min.

The effect of alginate on phagocytosis of latex particles

Alginate inhibited phagocytosis of latex particles in a dose-dependent fashion. When the macrophages were pre-incubated for 30 min, phagocytosis was inhibited ($P < 0{\cdot}05$) at 1 mg/ml and 2 mg/ml (Fig. 4). Inhibition was increased at all concentrations used when the alginate was added with the latex to the macrophages ($P < 0{\cdot}05$) at 0·25 mg/ml and 0·5 mg/ml and $P < 0{\cdot}01$ at 1 mg/ml and 2 mg/ml (Fig. 4).

DISCUSSION

Chronic lung infection is a major problem in patients with CF, although systemic infection is rare. The fact that these patients are immunologically competent and have high levels of circulating antibody against the infective bacteria (di Sant'Agnese & Davis, 1976; Høiby & Wilk, 1975) suggests the possibility of local impairment of immunity in the lung. Studies on alveolar macrophages from CF patients have been contradictory. Thomassen *et al.* (1980) showed that there was no intrinsic cellular defect of alveolar macrophages, while other reports showed them to have a decreased capacity to phagocytose candida (Cole, 1979; Sordelli *et al.*, 1982) or an increased phagocytic capacity (Cassino *et al.*, 1980). It has been claimed that the pathophysiology of lung disease in cystic fibrosis is the result of a genetically inherited metabolic defect in the monocyte/macrophage population (Wilson & Fudenberg, 1982).

The respiratory tract in CF offers an environment conducive to the production of mucoid *Ps. aeruginosa* (Macone *et al.*, 1981). As mucoid strains are rarely eliminated production of alginate may confer a selective advantage by protecting the bacteria from the host's immune response. It has been reported that mucoid *Ps. aeruginosa* survive more effectively in the pulmonary environment than their non-mucoid revertants (Govan, Fyfe & Baker, 1983) and that non-mucoid forms are more susceptible to phagocytosis than mucoid forms (Gosciniak *et al.*, 1980). Alginate has been shown to inhibit phagocytosis of bacteria by rabbit neutrophils (Schwartzmann & Boring, 1971) and similar results have been obtained with guinea-pig alveolar macrophages (Ruhen *et al.*, 1980).

An important function of alveolar macrophages is the elimination of foreign particles from the lung by phagocytosis (Green & Kass, 1964). Our studies show that phagocytosis is inhibited when the macrophages are pre-incubated with alginate. Inhibition was increased when alginate was added with the bacteria and decreased when the cells were vigorously washed to remove excess alginate. This suggests that the alginate was coating the macrophage and acting as a barrier to prevent attachment and phagocytosis. It is unlikely that the effect is due to impurities in the preparation. Alginic acid, a chemically similar compound showed a similar inhibitory effect. Further evidence for the barrier effect of the alginate comes from our studies on the binding of sensitized sheep erythrocytes to Fc receptors and phagocytosis of latex particles. Both binding and phagocytosis were inhibited by the alginate supporting the view that the alginate binds non-specifically to the macrophage and prevents particle attachment. Greater inhibition was observed when the alginate was added along with the indicator cells. The test particles may be trapped by the mucoid material and prevented from coming into contact with the macrophage as a result of its viscous nature.

Opsonization of bacteria facilitates phagocytosis and any impairment of this immune defence mechanism would be advantageous to the bacteria. The alginate from mucoid *Ps. aeruginosa* was shown to inhibit opsonization by specific antibody of the non-mucoid revertant. In fact at high concentrations of alginate opsonization was almost totally blocked. This result conflicts with the work of Baltimore & Mitchell (1980), who found that opsonization of two non-mucoid strains, which required antibody for opsonization was not inhibited by the addition of mucoid material. However, they used a different technique for alginate separation and measurement of opsonization, which was not distinguished from phagocytosis.

Pseudomonas alginate has recently been shown to be immunogenic (Pier, Matthews & Eardley, 1983; Bryan, Kureishi & Robin, 1983). However, the evidence suggests that mucoid bacteria are not effectively opsonized and that such antibody may even be harmful if it forms immune complexes in CF sera (Berdischewsky *et al.*, 1980; Moss & Hsu, 1982).

If the *in vitro* inhibitory effect of alginate on phagocytosis and opsonization, reported in this study, occurs *in vivo*, it may partly explain the predominance of mucoid *Ps. aeruginosa* in CF. These effects are likely to exacerbate chronic infection with *Ps. aeruginosa* by protecting the bacteria from the host's immune defences.

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